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## Clinical and Experimental

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# BIOLOGICAL STUDIES WITH ARSENIC[76]

## IV. The Histopathologic Effect of Arsenic[76] Upon the Hematopoietic Tissues of Patients With Leukemia

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and William Neal, M.D.
Chicago, Ill.

### INTRODUCTION

THIS paper is the fourth of a series on the medical applications of arsenic ($As^{76}$), a radioisotope with a half-life of 26.8 hours and emitting energetic beta and gamma rays. In the first paper the method of preparation by pile irradiation of cacodylic acid was described.[1] The second paper[2] defined the distribution of arsenic in mammalian tissues after intravenous injection; most of the arsenic was concentrated in the spleen, liver, and kidney within twelve to 24 hours, and half of the injected arsenic was excreted by the kidney within seventy-two hours.[2] In the third paper, treatment of neoplastic hematopoietic disease with $As^{76}$ was shown to be about as effective as with any of the commonly used therapeutic agents in that remissions were usually induced in chronic leukemia and only rarely in subacute or acute leukemia or multiple myeloma; one case of metastatic carcinoma also failed to respond.[3]

The purpose of this paper is the investigation of the histopathologic effects of $As^{76}$ upon hematopoietic tissues including liver and spleen in order to determine its mode of action. Since the biologic effects of all ionizing radiation are essentially similar,[4] it is hoped that information obtained in the present study will be applicable to the problem of the mechanism of irradiation therapy. In addition, a review of the literature indicates the presence of an almost complete gap in our knowledge of the histopathology of the liver and spleen in untreated cases of leukemia or in incipient stages of this disease. Therefore, descriptions of the histopathology of the organs in untreated patients have been included wherever such a description would supplement or modify our present knowledge.

### MATERIAL AND METHODS

Table I lists the biopsies obtained from the various patients with the time intervals between treatment and biopsy, as well as the source of the biopsies. Except where noted, a pretreatment biopsy was obtained within twenty-four hours prior to the injection of $As^{76}$. A total of 61 marrow biopsies, 23 splenic biopsies, 11 liver biopsies, and 3 lymph node biopsies were obtained from 19 patients. Biopsies of liver, spleen, and lymph nodes were performed with the Vim-Silverman needle.[5]

From the Departments of Medicine and Surgery, University of Chicago, and Argonne National Laboratory.

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Received for publication, Nov. 6, 1952.

TABLE I

| PATIENT DOSE ORGAN | CASE UNIT NO. | INTERVAL BETWEEN TREATMENT AND BIOPSY | | |
|---|---|---|---|---|
| | | *A. Acute Leukemia* | | |
| R. L.<br>69 mc.<br>marrow | 1<br>460931 | 4 days<br>N.S.C. | 30 days<br>N.S.C. | 46 days<br>mitoses ↑ |
| | | *B. Subacute Myelogenous Leukemia* | | |
| V. B.<br>17 mc.<br>marrow | 2<br>438819 | 10 days<br>N.S.C. | | |
| 17 mc.<br>spleen | | 10 days<br>N.S.C. | | |
| L. B.<br>90 mc.<br>marrow | 3<br>454329 | 16 days<br>N.S.C. | | |
| J. G.*<br>13.5 mc.<br>12 mc.<br>marrow | 4<br>54159 | 21 hours<br>(after 13.5 mc.)<br>iron ↑ | 4 months<br>further ↑ in iron | |
| 35 mc.<br>liver | | 3 days<br>iron ↑<br>cellular infiltrate ↑ ? | 13 days<br>definite ↓ cellular infiltrate | 18 days<br>further ↓ in cellular infiltrate |
| | | *C. Chronic Myelogenous Leukemia* | | |
| W. M.<br>60 mc.<br>spleen | 5<br>349870 | 3 days<br>deg. of mature and immature cells ↑ | 8 days<br>cells ↓ ,<br>cords and sinuses clearer,<br>mitoses not ↓ | 8 months‡<br>sarcomatous change,<br>fibrosis ↑ |
| J. B.<br>32 mc.<br>marrow | 6<br>435343 | 6 months<br>N.S.C. | 6½ months<br>no mitoses | |
| 59 mc.<br>marrow<br>spleen | | 36 hours<br>mature cells ↓ ?<br>18 hours<br>deg. ↑ ? | | |
| G. M.†<br>69 mc.<br>36 mc.<br>marrow | 7<br>461855 | 4 days<br>N.S.C. | | |
| 69 mc.<br>36 mc.<br>spleen | | 18 hours<br>mitoses ↓ ?<br>deg. ↑ ? | 5 days<br>cells ↓ ?,<br>sinusoidal structure, easier to see? | 14 days<br>further ↓ cells? |
| A Mayer<br>40 mc.<br>marrow | 8<br>430411 | 22 days<br>deg. ↑ ? | 40 days<br>cells ↓<br>erbl. ↑<br>right shift ↑<br>mitoses unchanged | 4½ months<br>cells ↑<br>left shift ↑<br>erbl. ↓ |
| M. S.<br>30 mc.<br>marrow | 9<br>415771 | 4 months<br>N.S.C. | | |
| 54 mc.<br>marrow | | 10 days<br>N.S.C. | 2½ months<br>N.S.C.<br>erbl. ↑ | |

TABLE I—CONT'D

| PATIENT DOSE ORGAN | CASE UNIT NO. | INTERVAL BETWEEN TREATMENT AND BIOPSY | | | |
|---|---|---|---|---|---|
| | | *D. Subacute Lymphatic Leukemia* | | | |
| H. W.† 36 mc. 39 mc. marrow | 10 424699 | 7 days N.S.C. | 29 days erbl. ↑ mitoses ↓ | | |
| 36 mc. 39 mc. liver | | 7 days m.l. ↓ iron ↑ ? | 29 days no. 1 in portal tissue iron ↑ | | |
| E. P. 14 mc. | 11 | 6 days erbl. and gran. ↑ ? | | | |
| | | *E. Chronic Lymphatic Leukemia* | | | |
| H. S. 76 mc. marrow | 12 199390 | 5 months cells ↑ due to ↑ s.l., erbl. ↑ | 5½ months erbl. ↑ megak. ↑ | | |
| 76 mc. spleen | | 21 hours N.S.C. | 5 days cords and sinuses seen more clearly, cells ↓ | 25 days cells ↑ iron ↑ | 5 months iron ↑ |
| J. G. 80 mc. marrow | 13 76786 | 20 days N.S.C. | | | |
| 80 mc. spleen | | 14 hours mast cells ↑ ? deg. ↑ ? | 20 days N.S.C. | | |
| W. B. 30 mc. spleen | 14 458095 | 17 hours N.S.C. | | | |
| 30 mc. lymph node | | 17 hours N.S.C. | 4 days N.S.C. | | |
| N. F. 38 mc. marrow | 15 458099 | 13 days erbl. ↑ ? megak. ↑ ? | | | |
| spleen 38 mc. | | 17 hours white pulp nodules seen | 13 days N.S.C. | | |
| | | *F. Polycythemia Vera* | | | |
| A. S. 45 mc. marrow | 16 428244 | 9 days megak. deg ↑ ? | 4 months N.S.C. | | |
| | | *G. Multiple Myeloma* | | | |
| W. D.* 15 mc. 12 mc. marrow | 17 414711 | 5 days N.S.C. | 24 days N.S.C. | 3 months N.S.C. | 6 months N.S.C. |
| P. Z. 88 mc. marrow | 18 434373 | 3 days neutrophils and precursors ↑ ? | 7 months hematopoietic cells ↓ ? | 9 months N.S.C. | 10 months hematopoiesis ↓ iron ↑ |

TABLE I—CONT'D

| PATIENT DOSE ORGAN | CASE UNIT NO. | INTERVAL BETWEEN TREATMENT AND BIOPSY | | |
|---|---|---|---|---|
| 35 mc. marrow | | 5 days‡ iron ↑ hematopoietic cells ↓ | | |
| | | *H. Metastatic Carcinoma (from stomach)* | | |
| G. L. liver | 19 436200 | 20 hours no tumor tissue | 5 days no tumor tissue | 15 days N.S.C. |
| marrow | | 1 day N.S.C. | 8 days serous fat atrophy cells ↓ | |

↑ = increase
↓ = decrease
? = questionable
cells = total cellularity
N.S.C. = no significant change
erbl. = erythroblasts
l. = lymphocytes
m. l. = medium lymphocytes
megak. = megakaryocytes
deg. = degenerate cells and/or cellular debris

*Pretreatment biopsy five days prior to treatment with $As^{76}$

†Injections one week apart; interval measured from first injection.

‡No pretreatment biopsy within twenty-four hours of treatment.

Observations were made in both smears and sections, but it soon became apparent that the smears were too unreliable and variable to permit comparison of pretreatment and posttreatment biopsies, and accordingly all the observations are based upon study of tissues fixed in formol-Zenker, sectioned at 6 micra in nitrocellulose, and stained with hematoxylin-eosin azure II.[6]

## RESULTS

The importance of adequate control material became apparent early in this study. Repeatedly, foci of degenerating cells, complete absence of mitoses, or slight variations in cellularity or unusual ratios of cells to one another were encountered in the post-treatment tissues. These phenomena ordinarily would have been ascribed to the effects of treatment had they not also been found occasionally in pretreatment biopsies (Figs. 1 and 9*b*). Moreover, there was a tendency for the tissues of any one patient to accumulate iron and for the spleen to undergo a sarcomatous change with the passage of months and years, metamorphoses which could easily have been ascribed to each succeeding course of treatment,

Fig. 1.—Foci of degenerating granulocytes (*G'*), many mature neutrophil granulocytes (*G*), reticular cells (*R*), spleen, untreated chronic myelogenous leukemia, Case 6. (×1150.)

Fig. 2*a*.—Portal infiltration with leukemic tissue (*P.I.*), liver prior to third course of treatment, subacute myelogenous leukemia, Case 4. (×130.)

Fig. 2*b*.—Same patient, decreased portal leukemic infiltration (*P.I.'*), thirteen days after treatment. (×130.)

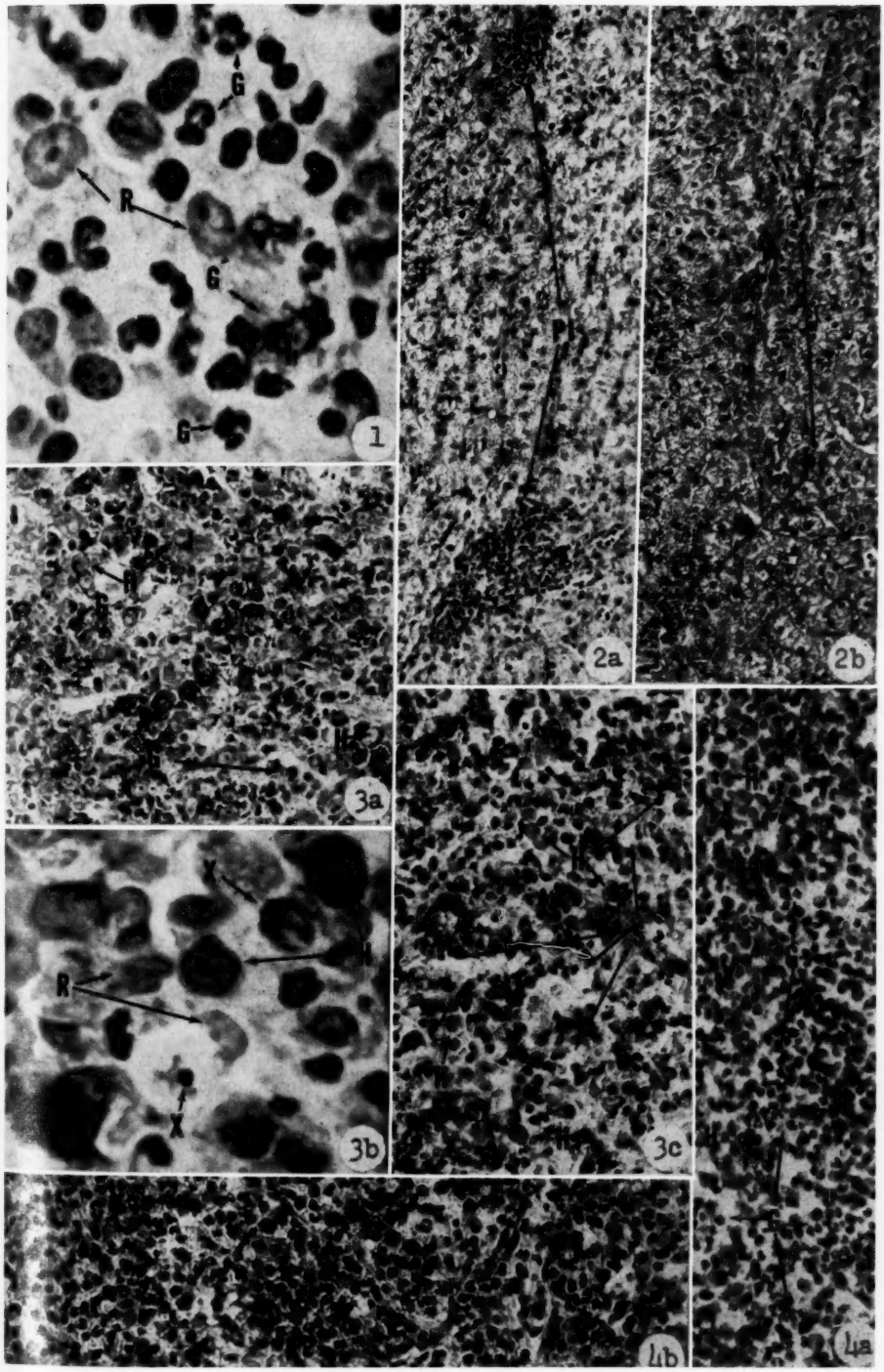
Fig. 3*a*.—Overwhelming of architecture by myelogenous tissue, mature granulocytes (*G*), as numerous as stem cells (*H*), spleen, untreated case of one and one-half year's duration, chronic myelogenous leukemia, Case 5. (×330.)

Fig. 3*b*.—Three days after treatment, diffusely spread degenerating cells (*X*), no evidence of selective degeneration of reticular cells (*R*) or hemocytoblasts (*H*), Case 5. (×1150.)

Fig. 3*c*.—Eight days after treatment, cords and sinuses (*S*) are clearer due to decrease in free cells, hemocytoblasts (*H*) still numerous. Architecture essentially similar to 3*a*, Case 5. (×290.)

Fig. 4*a*.—Overwhelming of architecture by myelogenous tissue with many clumps of adult granulocytes (*G*), occasional hemocytoblasts (*H*), spleen, untreated chronic myelogenous leukemia, Case 7. (×190.)

Fig. 4*b*.—Fourteen days after treatment, essentially similar to 4*a*, spleen, Case 7. (×190.)




*(For legend, see opposite page.)*

had not a biopsy been obtained prior to each new course. In general, it must be emphasized that the changes observed microscopically that could be ascribed to treatment were minimal and in no way proportional to the sometimes dramatic improvement in the peripheral blood and clinical course of some of the patients.

A. *Acute Leukemia.*—The marrow of the patient in Case 1, who failed to show any significant clinical or peripheral blood changes, except a drop in white count from 6,450 to 2,700, was studied at four, thirty, and forty-six days after treatment. This patient's marrow prior to treatment consisted of sheets of medium to large hemocytoblasts (myeloblasts) with rarely more mature marrow cells. Megakaryocytes were completely absent. The only change noted was a slight but probably significant increase in the number of mitoses at the time of death, forty-six days after treatment.

E. *Subacute Myelogenous Leukemia.*—Splenic, hepatic, and marrow tissues were studied in three previously untreated patients. The marrows of these patients were essentially similar, consisting of sheets of hemocytoblasts, promyelocytes, and myelocytes, with extremely rare erythroblasts and rare or absent megakaryocytes. Plasma cells lining arterial capillaries were fairly numerous. Abnormally numerous plasma cells were demonstrable repeatedly in this site in many marrow biopsies in all the patients both before and after treatment. Mitoses were not frequent, hardly more than the number seen in biopsies of marrows of patients without malignant disease.

The marrows of two of the patients (Cases 2 and 3) obtained at autopsy ten days and sixteen days, respectively, after treatment were essentially similar to the pretreatment biopsies, although the white count of Case 2 rose from 78,500 to 316,000 and that of Case 3 dropped from 8,400 to 1,050. The marrow of the patient in Case 4, who experienced a definite remission clinically and hematologically, failed to show any clear-cut effect of treatment at twenty-one hours and at four months after treatment except for a progressive increase in iron which was probably related to the 12 liters of blood he received during this period. His white count had dropped from 15,950 to 4,500 within one month of the double injection of $As^{76}$ (13.5 and 12 mc.) at which time more immature cells appeared in the peripheral blood.

In Case 2 splenic tissue prior to treatment consisted of a diffuse sheet of hemocytoblasts and very immature precursors of neutrophils and eosinophils in a reticular cell network. Mitoses were not unusual in number, and it was rare to find a cell with a multilobed nucleus. There was no white pulp, and cords and sinuses of the red pulp were not recognizable. Megakaryocytes and erythroblasts were not seen. At autopsy, ten days after 17 mc., in spite of an increase from 78,500 to 316,000 in the white count, the splenic tissue was similar to that obtained at biopsy.

Prior to the third course of treatment the major abnormality in the liver of the patient in Case 4 (Fig. 2*a*) was the presence of granulocyte precursors in the portal tissue. They showed the same degree of maturation as seen in the patient's marrow but in the liver hemocytoblasts were absent, suggesting a direct origin of promyelocytes from fixed tissue precursors. Iron was increased in the hepatic epithelium. Megakaryocytes, erythroblasts, and lymphocytes were not seen, and mitoses were extremely rare. The amount of infiltration was slight, being approximately equal to that seen in a subsiding hepatitis. The major abnormality was the presence of a single cell lineage rather than the number of cells.

At three days after treatment, no change except an increase in iron was noted, but by thirteen (Fig. 2*b*) and nineteen days after treatment, in parallel with a clinical remission and a drop in the white count from 11,700 to 3,800 within one month, a decrease in the amount of infiltrate in the liver was demonstrable without any evidence of a decrease in the number of mitoses or increase in the number of degenerating cells to account for the change. The differential count did not change appreciably during this remission. Four months after treatment the white count rose to 89,000 and the patient died.

C. *Chronic Myelogenous Leukemia.*—An unusually large number of splenic biopsies were available for study in this group because of the frequency of splenomegaly. A total of 10 serial biopsies were available from three patients (Cases 5 to 7) none of whom had satisfactory remissions. Prior to treatment (Figs. 1, 3*a*, and 4*a*) there was no distinction between red and white pulp, the spleen being replaced by a tissue consisting of a reticular cell network permeated with all the stages of maturation from the hemocytoblast to the adult granulocyte in both eosinophil and neutrophil series, similar to that seen in the marrow at a corresponding stage in the disease. Hemocytoblasts were no more numerous than adult granulocytes (Figs. 1, 3*a*, 3*b*, and 4*a*). Mitoses were not unusually frequent, and occasional clumps of degenerating adult neutrophils were seen (Fig. 1). In two patients (Cases 5 and 7) not even one mitosis was seen in some of the sections studied (Figs. 3*a* and 4*a*). White pulp was not demonstrable in these cases. In other untreated cases of chronic myelogenous leukemia we have occasionally seen small areas of white pulp. The almost complete absence at all stages of chronic myelogenous leukemia of erythroblasts and the extreme rarity of megakaryocytes and white pulp, as well as the uniform diffuse picture, differentiates chronic myelogenous leukemia from myeloid metaplasia.[7]

The patient in Case 5 experienced a four-month clinical remission characterized by a drop in his white count from 357,000 to 155,000 and a decrease in size of his spleen by 5 cm. within two and one-half weeks of treatment. At three days after treatment there was a definite increase in the amount of degeneration in both mature and immature forms (Fig. 3*b*), but mitoses were seen, whereas they were not seen in the pretreatment biopsy. Furthermore, this degeneration was not composed solely of foci of adult granulocytes seen commonly in untreated leukemic spleens (Fig. 1), but was more diffuse in nature (Fig. 3*b*). There was no evidence of any selective cytotoxic effect on the more immature cells (Fig. 3*b*). At eight days after treatment the spleen was more acellular so that the arrangement of the cords and sinuses was detectable. This decreased cellularity did not specifically involve the hemocytoblasts (Fig. 3*c*).

The spleen of Case 6 examined eighteen hours after injection of 59 mc. of $As^{76}$ (second course) had a questionable increase in degeneration in cells at about the myelocyte stage. However, his pretreatment biopsy had an unusually large amount of degeneration (Fig. 1). Certainly, there was no decrease in the number of mitoses. During his remission of about two months, the white count dropped from 143,000 to 55,000, and the spleen became about 4 cm. smaller.

Splenic tissue of the patient in Case 7 was examined at eighteen hours, five days, and fourteen days after the first of two injections of $As^{76}$. The biopsy eighteen hours after the first injection (69 mc.) failed to show any change except possibly a borderline increase in degeneration and a decrease in mitoses. The next had a borderline decrease in cellularity. There was also an increased number of adult granulocytes undergoing degeneration, but this was hard to evaluate since this is seen so commonly in untreated spleens of patients with chronic myelogenous leukemia. At fourteen days after the first injection and one week after a second injection of 36 mc., there was no further decrease in cellularity, and mitoses were present (Figs. 4*a* and 4*b*). The spleen became softer but no smaller. The white count dropped from 190,000 to 115,000 in this interval, with a decrease by half in the number of immature cells in the peripheral blood.

Noteworthy was the lack of change of architecture and the lack of decrease of immature cells following treatment in all cases (Figs. 3*a*, *b*, *c* and 4*a*, *b*). There was no evidence that irradiation decreased the number of immature cells and increased the number of mature cells as has been claimed by Émile-Weil[8] and Moeschlin.[9]

Biopsies of the spleen of a patient treated with urethane were examined to compare the effects of the two methods of treatment. At five days after initiation of urethane (1 gm. four times a day) which brought his count down to 12,000 from 245,000 and caused a decrease of about 5 cm. in the size of the spleen, there was no change in the microscopic

appearance of the spleen. Urethane was discontinued after a month of daily treatment, when the count was 12,000, and thereupon the patient experienced an exacerbation. Two weeks after this when the spleen had reached its pretreatment size and the white count 45,000, another biopsy was obtained. Thereafter x-ray therapy and four days later urethane were initiated, resulting in a reduction of the count to 13,000 with a reduction in the spleen by about 5 to 6 cm. Another biopsy of the spleen was obtained on the thirteenth day of x-ray therapy and the ninth day of the second course of urethane therapy. It failed to show any change except possibly a decreased cellularity. Each of the biopsies had distinctly more degeneration than seen in the spleen of any other patient in this study, and the excessive amount of degeneration seen in the three post-treatment biopsies would have been considered to be due to treatment if a control biopsy from the same patient had not been available for comparison.

The bone marrow prior to treatment consisted of dense hematopoietic tissue with 5 to 10 per cent fat. Granulocytes and their precursors constituted about 80 to 95 per cent of all of the cells, there usually being less than 10 per cent erythroblasts. All stages of granulocytopoiesis were present from hemocytoblasts to adult granulocytes, but with a greater preponderance of immature forms than seen in any other condition, except possibly splenic neutropenia or the regenerative stage following a toxic neutropenia.[10] However, the patient in Case 8, who had a remission of nineteen months, had as much of a right shift as seen in these two diseases (Fig. 5*a*). Mitoses were usually not strikingly increased. Also, as commonly seen in the other leukemic marrows, plasma cells were at least normal in amount and occasionally were found more frequently than normal, tending to be located along the arterial capillaries. Characteristically, in these comparatively early cases, megakaryocytes were increased in number, the only other condition in which they are more frequent being untreated polycythemia vera.

Case 8 is of unusual interest since after a comparatively small dose of 40 mc. she experienced a remission of nineteen months when last seen. At twenty-two days after treatment there was a slight increase in karyorrhexis which was probably not significant, but at forty days after treatment the marrow was less cellular and erythroblasts were increased (Fig. 5*b*). Foci of degenerating granulocytes (Fig. 5*b*) were seen in the latter biopsy but, as has been pointed out, this occurs sporadically in the untreated patients. By this time the white count had dropped from 124,250 to 15,000 with a return of the peripheral blood to about a normal differential. By four and one-half months after treatment the marrow was again similar to that seen prior to treatment. Nevertheless, the patient's remission continued about fifteen months longer.

The marrows of the other four patients examined, a total of 12 post-treatment marrows varying from eighteen hours to seven months after treatment, failed to show any significant change. In some of the patients there was a slight increase in erythroblasts and borderline changes in the number of degenerating cells, but invariably these changes were of such a minimal degree that examination of control pretreatment marrows usually disclosed similar phenomena. This group of tissues re-emphasized the need of adequate control material to serve as a basis of comparison to the post-treatment tissues. It is

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Fig. 5*a*.—Replacement of marrow by myelogenous tissue with cells varying from hemocytoblasts (*H*), to neutrophil granulocytes (*G*), with occasional erythroblasts (*E*), untreated chronic myelogenous leukemia, Case 8. (×345.)

Fig. 5*b*.—Forty days after treatment, decreased cellularity and increase in erythroblasts (*E*), degenerating granulocytes (*G'*), marrow, Case 8. (×345.)

Fig. 6*a*.—Numerous erythroblasts (*E*), among sheets of medium lymphocytes (*M*), untreated subacute lymphatic leukemia, marrow, Case 10. (×630.)

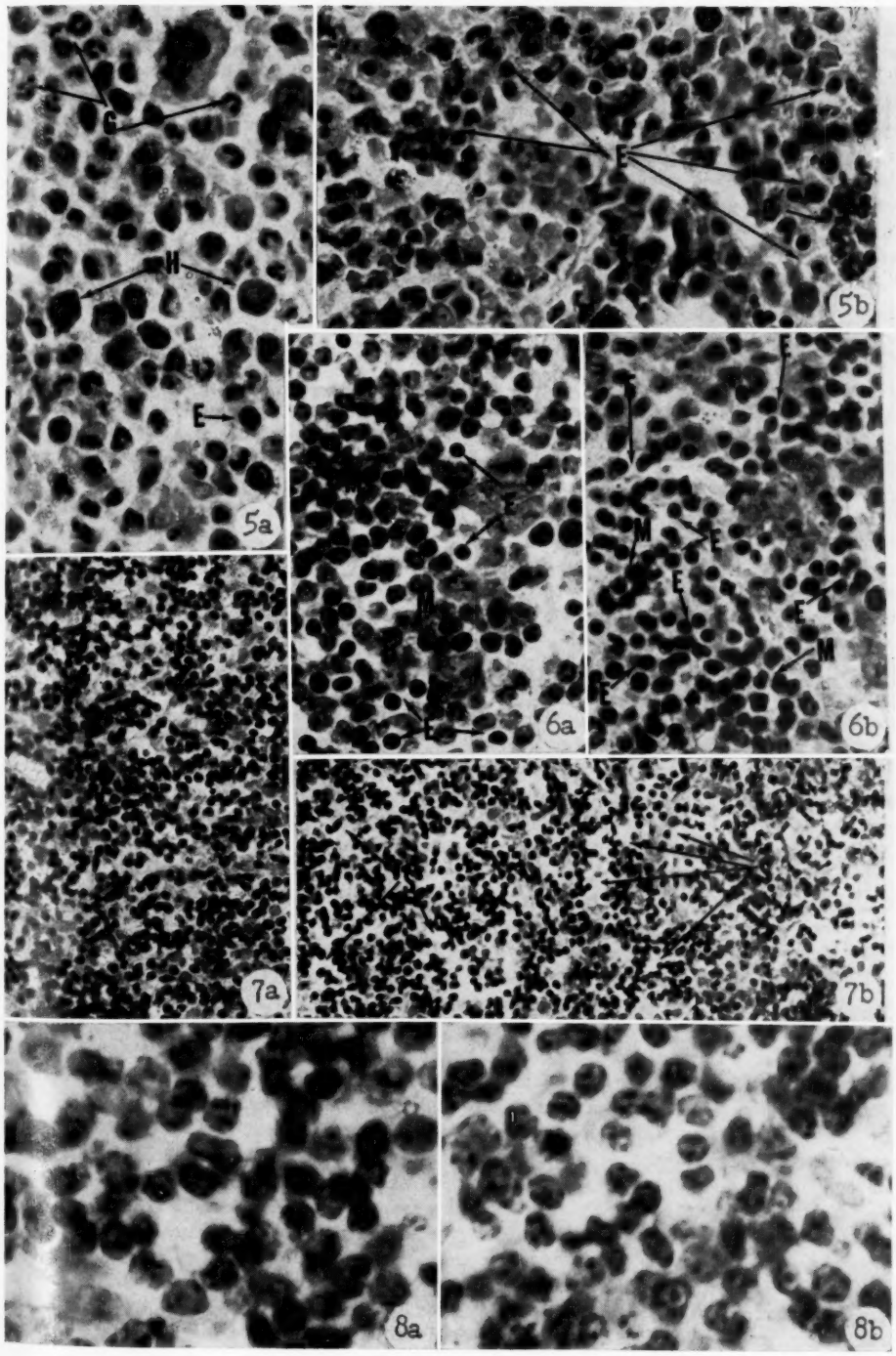
Fig. 6*b*.—Twenty-two days after second dose of $As^{76}$, marked increase in erythroblasts (*E*), and decrease in medium lymphocytes, (M), marrow, Case 10. (×630.)

Fig. 7*a*.—Diffuse sheet of small lymphocytes, pretreatment, chronic lymphatic leukemia, spleen, Case 12. (×400.)

Fig. 7*b*.—Five days after treatment, slightly decreased cellularity, cords and sinusoids (S) of red pulp recognizable, spleen, Case 12. (×325.)

Fig. 8*a*.—Diffuse sheet of lymphatic tissue, pretreatment, chronic lymphatic leukemia, spleen, Case 13. (×745.)

Fig. 8*b*.—Fourteen hours after treatment, essentially similar to Fig. 8*a*, spleen, Case 13. (×745.)




*(For legend, see opposite page.)*

noteworthy that the most marked changes were demonstrable in the marrow of the patient in Case 8, who had by far the most complete hematologic and clinical remission; her marrow prior to treatment was the most benign. It was surprising that the other patients, in particular Case 6 with a remission of two months and Case 9 with a remission of five months, failed to show anything significant. The marrow of the patient in Case 9 at two and one-half months after the second course of treatment was thought to have had a significant increase in erythroblasts coinciding with the height of her remission and a drop in white count from 105,000 to 7,100 and a decrease in the per cent of immature cells in the peripheral blood. This patient in the ensuing two years has developed severe chronic hemolysis and progressive increase in erythroblasts in the marrow making it questionable whether the increase in erythroblasts was due to $As^{76}$.

D. *Subacute Lymphatic Leukemia.*—The patient in Case 10 was of unusual interest in that he had a severe associated hemolytic anemia. This was reflected in his bone marrow, in which erythroblasts were almost as numerous as medium lymphocytes (lymphoblasts), something never found in any of our patients with untreated uncomplicated leukemia (Fig. 6*a*). Mitoses were frequent in these erythroblasts. The liver, however, presented the usual picture of subacute lymphatic leukemia with infiltrates of monomorphous lymphocytes of medium size in the portal tissue and complete absence of erythroblasts, megakaryocytes, and granulocyte precursors, superficially resembling subacute hepatitis, but the pleomorphism characteristic of the inflammatory response in the latter was completely lacking. There was an excess of iron pigment in the portal tissue, Kupffer cells, and parenchyma cells, the typical picture of hemosiderosis.

Seven days after 36 mc. of $AS^{76}$, the marrow was essentially unchanged. Another 39 mc. of $As^{76}$ was injected at that time and the marrow examined twenty-two days later, at which time the erythroblasts were greatly increased in number (Fig. 6*b*).

The liver had a marked decrease in lymphatic infiltration seven days after treatment, and twenty-two days later there was still no lymphatic regeneration. This decrease in infiltration paralleled the decrease in white count (94,500 to 16,250), increase in percentage of neutrophils from 30 to 48; and increase in erythroblasts in the marrow. It was similar to that noted in the liver of the patient in Case 4 following treatment (Figs. 2*a* and 2*b*).

The other patient in the group, in Case 11, had a definite remission in fever, size of nodes, and a drop in the white count from 34,700 to 11,750 but without any change in differential count. This beneficial effect, following only 4 mc., what we now consider a homeopathic dose, was accompanied by a questionable increase of a minor degree in erythroblasts and granulocytes in the bone marrow at six days after treatment. It seems most odd that so inadequate a dose could cause histopathologic and clinical improvement when other patients receiving six times as much $As^{76}$ failed to show any effect.

E. *Chronic Lymphatic Leukemia.*—Prior to treatment the spleens (Figs. 7*a* and 8*a*) of the 4 patients were essentially similar, consisting of a dense sheet of lymphatic tissue greatly resembling the dense diffuse internodular tissue seen in the cortex of a normal lymph node. Because of the cellularity, it was extremely difficult to recognize any landmarks except the connective tissue trabeculae. There was no myeloid metaplasia. The lymphocytes were almost exclusively of the small variety. Occasionally, one noted some phagocytized iron pigment. Mitoses were quite rare, hardly more frequent than in a nonleukemic spleen.

Case 12 merits special attention since this patient experienced a five-month remission terminated by death due to congestive heart disease. During this time he maintained a normal hemoglobin after only two transfusions at the time of treatment and seven transfusions in the following month. Previously, massive transfusions (approximately 10 to 20 per month) accompanied by a wide variety of therapies barely served to maintain a level between 4 and 9 Gm. of hemoglobin. Fortunately, this patient had two splenic punctures, one a few hours prior to $As^{76}$ treatment, and the other four months earlier to serve as control material for the 3 biopsies and autopsy specimen of the spleen. A comparison of the pretreatment specimens indicated the variability present in the control material since one biopsy had the same amount of pyknosis as seen at twenty-one hours after treatment, whereas the other had none at all (Fig. 7*a*). It is probable that had only one biopsy been

available before treatment, the pyknosis at twenty-one hours after treatment would have been considered due to the effect of the $As^{76}$. By five days after treatment there was a slight decrease in the number of small lymphocytes so that the red pulp could be differentiated from the white pulp and the cords and sinuses were slightly more easily recognizable (Fig. 7*b*). However, by twenty-five days after treatment, the cellularity had increased again and at autopsy, the cellularity had returned to its pretreatment level. There was a gradual increase in iron pigmentation. This histopathologic response in the spleen paralleled but was much less clear-cut than the obvious decrease in size of the spleen and nodes and marked subjective improvement. The white count dropped from 298,000 to 91,000 within one month after treatment only to return to its original level by the time of the patient's death. At this time his hemoglobin was still 13 Gm.

The other patients (Cases 13 to 15), because of various complications, failed to live long enough to assess the effect of therapy clinically. All experienced either a 30 per cent drop in white count or moderate decrease in size of their spleens. Splenic tissue examined at seventeen hours (Case 14), seventeen hours and three days (Case 15), and fourteen hours and twenty days (Case 13) failed to reveal any significant change compared to the control splenic tissue (Figs. 8*a* and 8*b*). The importance of the occasional nodules of white pulp seen in the spleen of Case 15 at seventeen hours is hard to evaluate since they are seen sporadically in chronic lymphatic leukemia. The spleen of Case 13 at autopsy had large areas of necrosis, but it is impossible to determine whether or not these had existed prior to treatment and, if not, was this necrosis ascribable to treatment or did it occur as part of the natural history of his disease.

Through the cooperation of the x-ray therapy department a patient with untreated lymphatic leukemia was given through a portal of 10 cm. by 10 cm. over the spleen one dose of 600r measured in air representing a depth dose of 408r. A biopsy of the spleen in the center of the portal was obtained immediately prior to as well as twenty hours after treatment. Examination of these two specimens failed to reveal any significant difference between the two.

Prior to treatment the marrows of all the patients except that of Case 12 were almost solidly cellular, practically all cells being small lymphocytes. This patient's marrow had about 15 per cent fat, a most unusual finding in chronic lymphatic leukemia. The patient in Case 13 had an unusually large number of megakaryocytes in his marrow.

The marrow of the patient in Case 12 was the only one to show any significant change, an increase in the number of erythroblasts, megakaryocytes, and granulocyte precursors, as well as in iron pigment at five and five and one-half months after treatment corresponding to his clinical remission. During this time the differential did not improve, but the platelet count increased from 60,000 to 100,000, and the white count dropped from 294,000 to 150,000. The marrow of the patient in Case 13 at autopsy at twenty days after treatment had large areas of spontaneous necrosis similar to those seen in his spleen. It was impossible to determine if these areas were due to treatment or had occurred spontaneously.

In Case 14 lymph node tissue obtained prior to treatment, as well as at seventeen hours and again at four days after injection of 38 mc. of $As^{76}$, were practically identical with that seen in the patient's spleen. During this time the patient's white count dropped from 372,000 to 251,000. Further observation was impossible because he died of a cerebral hemorrhage.

F. *Polycythemia Rubra Vera.*—Examination of the bone marrow of the one patient treated with 45 mc. of $As^{76}$ failed to reveal any change at nine days and at four months after treatment. It is probable that he had an insufficient amount of $As^{76}$, and his remission was due to phlebotomies prior to treatment with $As^{76}$. However, both his white count and platelets diminished by about 30 per cent so that he must have had some effective irradiation. After treatment with $P^{32}$ his marrow fat quadrupled, a characteristic noted in all patients with polycythemia vera undergoing a remission following $P^{32}$.[11]

G. *Multiple Myeloma.*—These two patients had clinical manifestations and histopathology that represented in one (Case 17) a most benign type and in the other (Case 18) an extremely malignant type of multiple myeloma. The patient in Case 17 continued to

show a remission after various other therapies including x-ray therapy and symptomatic treatment. His bone marrow has been examined 13 times during which the variation has been greater than that seen between the pretreatment biopsy and the biopsies at five days after the first injection, and seventeen and eighteen days, and six months after the second injection. In fact, in about two-thirds of all the biopsies this patient had it was impossible to make the diagnosis of multiple myeloma because of the sparsity of plasma cells. This case illustrates the extreme difficulty of attempting to assess the effect of treatment even with supposedly adequate control material where the lesion is spotty in nature, minimal in extent, and in which there may be a gradual change inherent in the natural history of the disease.

The other patient (Case 18) presented an entirely different marrow picture, the more common appearance of multiple myeloma. The pretreatment marrow was composed of islands of plasma cells with only occasional normal marrow cells. Fat cells represented 40 per cent of the marrow. In spite of the fact that this patient had experienced a dramatic clinical remission, biopsies at three days, seven months, and nine months after treatment failed to show any significant change. At ten months plasma cells had increased and hematopoietic cells decreased.

He was then given another injection of 35 mc. of $As^{76}$ without any clinical response. Examination of the marrow five days after the second injection showed a decrease in the number of hematopoietic cells, although it is possible that this change might have preceded the second injection since no pretreatment biopsy was available. There was a progressive increase in iron storage during the eleven months that the patient's marrow was examined, probably due to repeated transfusions.

H. *Metastatic Carcinoma.*—This case is of more than usual interest because examination of the liver biopsies once again illustrated the difficulty inherent in the study of the histopathologic effect of therapy in what would seem adequate material. The diagnosis of metastatic carcinoma was not suspected since the patient was admitted with a diagnosis of hepatosplenomegaly of unknown etiology. What was thought to be spleen was biopsied by a transabdominal approach. Examination of the tissue revealed liver tissue with metastatic carcinoma. His liver was biopsied twenty hours, five days, and 15 days after treatment, and hepatic tissue was also obtained at autopsy. Two of the post-treatment biopsies did not contain any tumor tissue. Examination of the hepatic tissues obtained at autopsy revealed such a wide variety of stages in the growth and degeneration of the islands of tumor tissue as to make it impossible to draw any valid conclusions relative to the changes seen in the various needle biopsies which did contain tumor tissue.

Two biopsies of his marrow prior to treatment disclosed tissue which was 55 to 60 per cent hematopoietic with an essentially normal distribution of cells except for a maturation arrest in the granulocyte series (Figs. 9*a* and 9*b*). Foci of degenerating neutrophil granulocytes were seen (Fig. 9*b*) but, as already described in this paper and in other material we have encountered, this is a sporadically but widely occurring phenomenon in sections of all types of human marrows.[10] One day after treatment the marrow was unchanged, but eight days later it had undergone an extensive serous fat atrophy with a decrease in mitoses and a reduction by about one-third in cellularity (Fig. 9*c*). The maturation arrest of the granulocyte series was still present (Fig. 9*d*). This decreased marrow activity was not reflected in the peripheral blood since the patient's white count increased from 9,000 to 23,000 and his platelet count remained stationary.

## DISCUSSION

This study was completely lacking in one important aspect—failure to study tissue from the overflow areas of the marrow, areas which are cellular in leukemia and comparatively atrophic in the normal individual. It is possible that such areas might be the sole site of dramatic change following irradiation. Another limitation was the comparatively small size of the biopsies, especially the marrow biopsies, the latter being obtained by sectioning particles of marrow aspirated

through a nine-gauge needle. Fortunately, the marrow,[12] spleen, nodes, and, to a lesser extent, the liver are each quite uniform in structure in leukemia, partially compensating for this difficulty. Inability to obtain biopsies from comparable sites was an insurmountable obstacle in the study of the liver in the single case of metastatic carcinoma (Case 19) and perhaps in the marrow in the comparatively benign case of multiple myeloma (Case 17).

There are certain explanations for our inability to demonstrate any marked histopathologic effects following treatment with arsenic[76] even in patients undergoing a clear-cut clinical remission. At a median lethal dose of total body x-

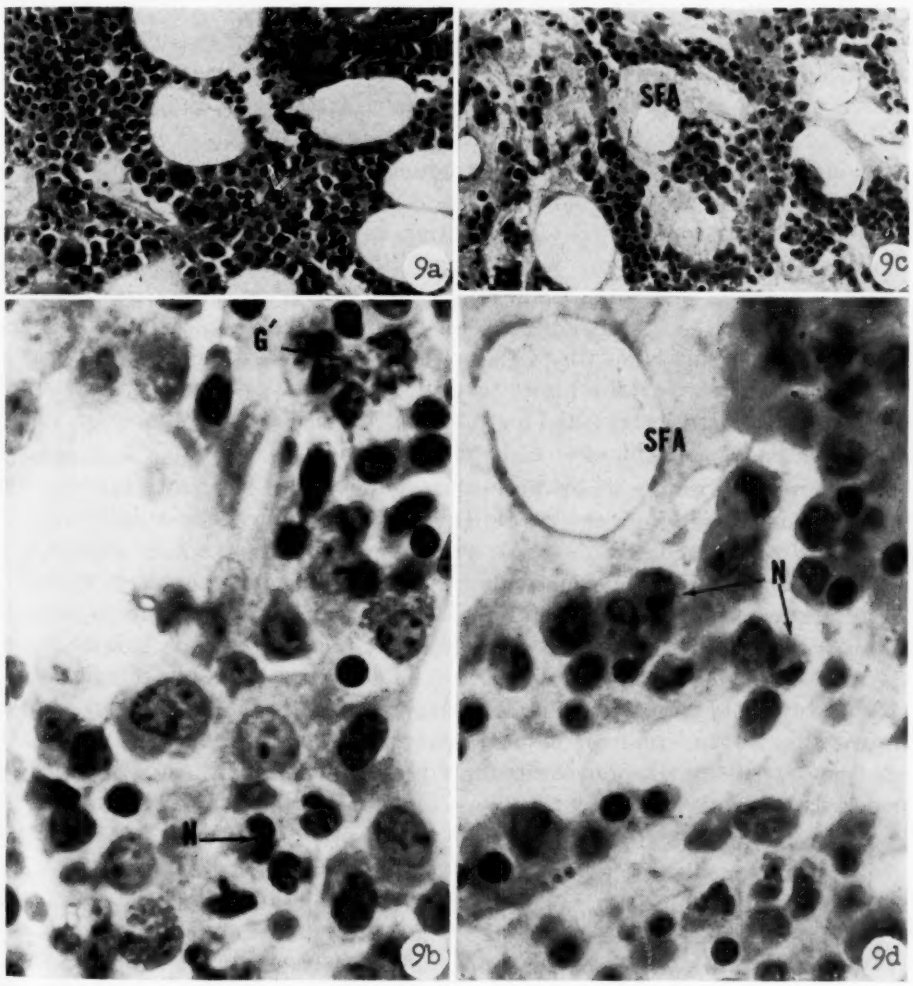



Fig. 9*a*.—Pretreatment marrow, normal cellularity, Case 19. (×225.)

Fig. 9*b*.—Maturation arrest at metamyelocyte stage (*N*), degenerating mature granulocytes (*G'*), same marrow as Fig. 9*a*. (×1150.)

Fig. 9*c*.—Eight days after treatment, decreased cellularity and serous fat atrophy (*SFA*), Case 19. (×225.)

Fig. 9*d*.—Higher power of Fig. 9*c*, serous fat atrophy (*SFA*) and maturation arrest at metamyelocyte stage (*M*), same marrow as Fig. 9*c*. (×1150.)

irradiation or neutron irradiation extreme necrosis of susceptible cells (erythroblasts, myelocytes, megakaryocytes, and small lymphocytes), phagocytosis of debris, aplasia, and finally regeneration may be demonstrated unequivocably. When intensity of total body irradiation is reduced to about one-tenth to one-eighth of the median lethal dose it is impossible to demonstrate any microscopic evidence of changes produced in the hematopoietic tissues in spite of the fact that a drop in the peripheral blood count occurs.[4, 13]

An analysis by Dr. Marinelli of the Argonne National Laboratories of the intensity of irradiation after injection of $As^{76}$ reveals that a dose level equivalent to 50 to 125r of total body x-irradiation was attained. This is approximately equal to the level of total body x-irradiation in the experimental animal in which histopathologic evidence of irradiation is not demonstrable. From the animal experiments already alluded to, it is clear that changes in the peripheral blood and size of hematopoietic organs may be found without histopathologic evidence of radiation destruction, and it is not surprising that a similar situation existed in our patients. Furthermore, an inability to demonstrate any microscopic effect of irradiation on hematopoietic tissues does not signify that there is no functional effect. Hennessy and Huff[14] showed a decreased uptake of radioiron following doses as low as 5r of total body x-irradiation in rats, a level of irradiation less than a tenth of that which yields detectable microscopic evidence of tissue damage. Their study demonstrates a reduced capacity of erythroblasts to utilize radioiron without differentiating between a decrease in normal function or a decrease in the number of viable erythroblasts, or a combination of both possibilities.

Following injection of $P^{32}$ and other radioisotopes in animal experiments it may be impossible to demonstrate any degeneration of cells or inhibition of mitoses to account for the depression of the peripheral blood, decrease in size of and microscopically demonstrable aplasia of the hematopoietic tissues.[15] This does not mean that internal irradiation does not cause cellular degeneration and mitotic inhibition, but probably that these destructive processes are not marked enough at any one instant to be demonstrated by histologic study. Because of the prolonged effect of isotopic irradiation, a summation of continued slight submicroscopic damage probably occurs which finally results in aplasia, decrease in size, and lowered production of cells by the marrow, spleen, nodes, and thymus.

In order to evaluate the literature on the subject of histopathologic effects of irradiation on the tissues of leukemic patients, we have set up the following criteria based on our experience in this study. An adequate biopsy of the organ in question must be available immediately prior to treatment, and further serial biopsies should be available for comparison. Enough follow-up material should be available to insure that any changes demonstrable are due to treatment and not due to changes occurring as part of the natural history of the disease. The tissues studied must be prepared by a technique which is in itself not inherently susceptible to extreme variations such as dry smears of sternal aspirations. The lesions must be fairly uniform and not spotty as seen

in some cases of multiple myeloma or metastatic carcinoma, and preferably should not differ from node to node as may occur in Hodgkin's disease.

Utilizing these criteria, we have been able to find a few papers in which irradiation has been proved to cause definite microscopic changes in the tissues of patients with lymphomas. Windholz[16] irradiated the lower pole of the spleen of a patient with chronic myelogenous leukemia, the patient dying in fourteen hours. He noted degeneration of free cells, swelling of reticular cells, and decreased cellularity, and stressed the similarity of these findings to those seen after irradiation of normal splenic tissue. Similar acute necrosis has been described in Hodgkin's disease,[17, 18] in lymphatic leukemia,[19] and in mycosis fungoides.[20, 21] In all these cases large doses of x-ray therapy directed through one portal were used and the changes were all produced within a few hours or at most two to three days after treatment and were not the result of continuous low intensity isotopic irradiation. There is agreement on three points as regards irradiation of neoplastic hematopoietic tissues: the cytotoxic effects are limited largely to within twenty-four hours after irradiation; irradiation does nothing to alter the basic nature of the disease process; and, there is no histologic evidence to prove that neoplastic cells are any more susceptible than normal cells. Certainly, the widely quoted statement originally made by Bergonié and Tribondeau,[22] based on the most meager evidence, that rapidly dividing or immature cells are more susceptible to irradiation than more normal cells has never received any experimental support as far as blood-forming tissues are concerned and has again been challenged.[23, 24] In the present study there was no greater necrotizing or inhibitory effect of the $As^{76}$ upon immature than mature cells.

We are not aware of any studies on the effect of radioisotopes which meet the above criteria. However, a similar situation exists following nitrogen mustard and urethane therapy, both of which exert as diffuse but not as prolonged an effect as does $As^{76}$ or $P^{32}$. From a review of the literature, as well as from our own material,[13] we have concluded that nitrogen mustard induces slight degeneration or no demonstrable degeneration when used in therapeutic doses, a concept since corroborated,[25, 27] although at median lethal doses in animals an intense necrotizing effect is readily demonstrable.[13, 28] A decreased cellularity is usually demonstrable in the skin lesions of mycosis fungoides following therapeutic doses of nitrogen mustard.[29, 30] Similar results are achieved with the use of urethane in mycosis fungoides.[31]

It is therefore clear that the failure to demonstrate anything but the inconsistent presence of a slight amount of cellular degeneration and aplasia in patients receiving therapeutic doses of $As^{76}$ and even in patients responding to this treatment with remissions is not unique. In general, similar phenomena has been encountered after treatment with nitrogen mustard, urethane, and in some cases x-ray therapy.

Perhaps even more fundamental are the characteristics common to all of these agents; that none change the basic nature of the histopathologic lesion; that all in median lethal doses are cytotoxic to the same cell types (small lymphocytes, erythroblasts, myelocytes, and megakaryocytes), inhibit mitoses

and, except in extremely high doses, spare the most fundamental cell in hematopoiesis, the reticular cell. Furthermore, when one considers the mechanism of regeneration, homoplastically from surviving susceptible cells and heteroplastically from reticular cells, as well as the fact that a specific destructive effect has never been demonstrated on malignant as contrasted to nonmalignant hematopoietic cells or upon the reticular cell as opposed to more mature cells, one cannot help but agree with Bloom[32] that it is extremely doubtful that irradiation or any similar technique will on theoretical grounds ever result in a cure of neoplasms of the hematopoietic tissues. There is no lack of clinical experience to support this viewpoint.

## CONCLUSIONS

Emphasis is placed upon the necessity of well-controlled material to assay the histopathologic mechanisms of reaction to irradiation or chemotherapy, and in particular not to ascribe to treatment phenomena which are incidental or part of the natural history of the disease.

At the level of irradiation dosage following injection of $As^{76}$ occasionally evidence of cellular destruction, in some cases followed by decreased cellularity, may be demonstrable.

In general, there is either no evidence or insufficient evidence of cytolysis or inhibition of cellular proliferation to account for the remissions produced. There is no evidence that immature rapidly dividing cells are more sensitive, or that irradiation by $As^{76}$ induces a right shift in benign or neoplastic hematopoietic tissues.

Review of the literature reveals similar experience with nitrogen mustard.

The concept is presented that irradiation in particular and cytotoxic therapy in general will probably not achieve a cure of these diseases.

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# THE RATE OF FORMATION OF NONDIFFUSIBLE (ORGANIC) FRACTION OF $I^{131}$* IN PLASMA CORRELATED WITH APPARENT THYROID STATE

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## INTRODUCTION

THE finding of an accurate diagnostic test for thyroid function has long been a problem. The basal metabolic rate[1] is not always a measure of thyroid function. The chemical method[2-5] of determining plasma protein-bound iodine is laborious and subject to error. Radioiodine is also used to measure thyroid function by observing the rates of urinary excretion[6-8] in vivo gland uptake[9-12] and the conversion of inorganic into plasma protein-bound radioiodine.[13-16] All have added to the knowledge of thyroid physiology, but in each case discriminating diagnostic information necessary for routine clinical use was incomplete either because of combined variables[17] or because of partial application of the method.

To achieve further discriminating diagnostic information, the erythrocyte/plasma $I^{131}$ ratio[18] as previously used in this laboratory has now been used for calculating the per cent of nondiffusible (organic) radioiodine of the plasma at two specific time intervals following the oral administration of inorganic radioiodine ($I^{131}$).

Maximum differentiation between apparent hyperthyroid and euthyroid states was observed at twenty-four hours, whereas maximum differentiation of hypothyroid from euthyroid states is observed at seventy-two hours.

## MATERIALS AND METHODS

This method was expanded by studying 40 patients referred by Medical Services for thyroid state evaluation who had clinical diagnoses of hypothyroidism or hyperthyroidism which was based on the usual classical signs and symptoms as observed by changes in the following: nervous state, eye, pulse, weight, gastrointestinal, and cardiovascular; size of thyroid gland; condition of skin; tremor or its lack; anemia, etc. The laboratory criteria used were basal metabolic rate, blood cholesterol, and protein-bound iodine.

A euthyroid† group of 70 cases was also selected from those members of the hospital patient population who showed no symptoms or signs suggesting thyroid abnormality, due regard being given to extrathyroid conditions which might alter thyroid function, such as nonthyroid disease[19] or the ingestion of antithyroid medications or iodinated compounds[20] or thyroid hormone.[21] Included in this group were several cases of nontoxic colloid goiters.

## ERYTHROCYTE/PLASMA $I^{131}$ RATIO DETERMINATIONS

Four hours after 100 to 150 $\mu$c of $I^{131}$ by mouth, 5 ml. of heparinized blood is drawn and centrifuged in 15 ml. tubes at 2,000 times gravity for thirty minutes. Similar

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*The radioiodine used in these studies was supplied by Oak Ridge National Laboratory on authorization from the Isotope Division, U. S. Atomic Energy Commission.

†The term "euthyroid" designates those whose tissues at large receive the normal amount of hormone daily, irrespective of the anatomic state of the thyroid gland.[22]

samples were taken after one, two, three, and four days in order to determine the intervals necessary for maximum discrimination of apparent thyroid state. Samples of red cells and plasma, 2 Gm. each, are transferred separately with 3 ml. Luer-Lok syringes with 18-gauge spinal needles to aluminum dishes (4 cm. diameter) and gamma activity measured wet by scintillation counting using a sodium iodide thallium activated crystal. Pulse height cutoff was raised until background (well shielded) was reduced to less than two counts per second. Net sample counts ran from 20 counts per second in four-hour samples to 0.1 count per second at four days. Counting efficiency was estimated at 8 per cent of total ($4\pi$) disintegrations.

The activity of the plasma includes inorganic iodide and organic iodine bound or not. Only the inorganic iodide ($I^{131}$) enters the red cells. Water space differs in the two compartments,[23] and the equilibrium* which is rapidly attained[24] is not 1:1 but 0.6 in red cells to 1.0 in plasma.[18, 25] With the passage of time, iodide is excreted, lowering the activity in both compartments and the thyroid builds new ($I^{131}$-labeled) thyroxine,[26, 27] which appears in the plasma but does not diffuse into the red cells. This has been also observed by other investigators.[28-30]

Using the $I^{131}$ activity of the red cells to estimate the iodide activity in the plasma, the over plus of plasma activity is obviously organic iodine. At any time, the plasma compartment activity is the sum of diffusible $I^{131}$ (presumably iodide ions) and nondiffusible (organic) $I^{131}$. The diffusible has an equilibrium ratio, red cells vs. plasma of 0.6. The per cent of nondiffusible radioiodine of the plasma was calculated on this basis (see Table I).

TABLE I. R.B.C./PLASMA $I^{131}$ RATIO VS. PERCENTAGE OF NONDIFFUSIBLE PLASMA $I^{131}$

| R.B.C./PLASMA $I^{131}$ RATIO | PERCENTAGE OF NONDIFFUSIBLE PLASMA $I^{131}$† |
|---|---|
| 0.6* | 0 |
| 0.57 | 5 |
| 0.54 | 10 |
| 0.51 | 15 |
| 0.48 | 20 |
| 0.45 | 25 |
| 0.42 | 30 |
| 0.39 | 35 |
| 0.36 | 40 |
| 0.33 | 45 |
| 0.30 | 50 |
| 0.27 | 55 |
| 0.24 | 60 |
| 0.21 | 65 |
| 0.18 | 70 |
| 0.15 | 75 |
| 0.12 | 80 |
| 0.09 | 85 |
| 0.06-0.03‡ | 90-95§ |

*Ratio value at which there was no nondiffusible $I^{131}$ present.

†See method for basis of these calculations.

‡Limits of centrifugal separation of blood leaving a slight contamination of the R.B.C. with nondiffusible plasma $I^{131}$.

§Corrected for estimated 2 per cent contamination of red cells with nondiffusible activity.

## RESULTS

Table II summarizes the results from the red cell: plasma ratios and the ratios expressed as per cent of nondiffusible plasma radioiodine in 30 hyperthyroid patients, 70 euthyroid subjects, and 10 hypothyroid patients. Note that the calculated per cent of nondiffusible plasma $I^{131}$ increases very rapidly to over 90 per cent in many of the hyperthyroid cases and

*This apparent radioiodide equilibrium between the R.B.C. and plasma was observed within a few minutes following I. V. injection and within a few hours following oral ingestion of radioiodide.

reaches at least 70 per cent in all of them. Some of the cases called euthyroid also reached 90 per cent, but not until the fourth day, and some showed little or none during the first few days following the oral administration of inorganic $I^{131}$. The hypothyroid cases showed little or no nondiffusible plasma $I^{131}$ even in four days.

TABLE II. RATIO OF ACTIVITIES OF RED BLOOD CELLS AND PLASMA, WITH CALCULATED PERCENTAGE OF NONDIFFUSIBLE PLASMA $I^{131}$ AT VARIED INTERVALS FOLLOWING THE ORAL ADMINISTRATION OF INORGANIC $I^{131}$

| | | | 4 HR. | 24 HR. | 48 HR. | 72 HR. | 96 HR. |
|---|---|---|---|---|---|---|---|
| HYPERTHYROID 30 CASES | R.B.C./plasma ratio | Mean | 0.52 | 0.16 | 0.11 | 0.09 | 0.09 |
| | | Range | 0.65-0.26 | 0.40-0.06 | 0.33-0.04 | 0.18-0.04 | 0.18-0.03 |
| | Calculated per cent of nondiffusible plasma $I^{131}$ | Mean | 13 | 73 | 83 | 85 | 85 |
| | | Range | 0-57 | 33-90 | 45-93 | 70-93 | 70-95 |
| EUTHYROID 70 CASES | R.B.C./plasma ratio | Mean | 0.58 | 0.56 | 0.46 | 0.28 | 0.21 |
| | | Range | 0.69-0.52 | 0.69-0.40 | 0.63-0.15 | 0.40-0.10 | 0.38-0.05 |
| | Calculated per cent of nondiffusible plasma $I^{131}$ | Mean | 3 | 7 | 23 | 53 | 65 |
| | | Range | 0-13 | 0-33 | 0-75 | 23-83 | 37-92 |
| HYPOTHYROID 10 CASES | R.B.C./plasma ratio | Mean | 0.60* | 0.59 | 0.59 | 0.60 | 0.60 |
| | | Range | 0.70-0.50 | 0.70-0.54 | 0.70-0.53 | 0.70-0.55 | 0.70-0.58 |
| | Calculated per cent of nondiffusible plasma $I^{131}$ | Mean | 0 | 1 | 1 | 0 | 0 |
| | | Range | 0-8 | 0-10 | 0-12 | 0-8 | 0-3 |

*Ratio value representing the absence of nondiffusible $I^{131}$.

Attention is now called to Fig. 1, a graphic presentation of Table II. Maximum separation of the hyperthyroid from the euthyroid state was observed at twenty-four hours, as range overlap between the two designated thyroid states was present before and following twenty-four hours. Maximum discrimination between hypothyroid and euthyroid states was not observed until seventy-two hours, when range overlap was finally eliminated.

## DISCUSSION

The per cent of nondiffusible plasma $I^{131}$ calculated from the ratios of activities of red cells and plasma appears to differentiate hyperthyroid cases from euthyroid most dependably at twenty-four hours. By twenty-four hours the elevated metabolic rate of the hyperfunctioning gland has practically converted all of the remaining blood iodide ($I^{131}$) to organic $I^{131}$; in contrast, at this time, there is usually little or no release of organic $I^{131}$ by the euthyroid gland. However, by forty-eight hours or later euthyroid function is evidenced by the continued conversion of inorganic $I^{131}$ and release as organic $I^{131}$.

Maximum discrimination of hypothyroid from euthyroid function was not observed until seventy-two hours due to the variable release of organic $I^{131}$ by the euthyroid gland. In about twenty per cent of the euthyroid cases the presence of nondiffusible plasma radioiodine was not observed until seventy-two hours. However, the delay in this group of twenty per cent appeared to be compensated by the fact that the amount of organic $I^{131}$ released was comparable to that shown by the remaining euthyroid cases exhibiting uniform release. Colloid goiters also showed this delay to an even more marked degree; their total release of organic $I^{131}$ in time appears to be within euthyroid limits but the delay also was at least three or more days.

It seems likely from the small series that this method is superior to gross thyroid uptake measurements and as accurate as the serum protein-bound method for obtaining routine clinical information regarding apparent thyroid states. Furthermore, the absence of chemical separations in this method eliminates additional variables thereby yielding results which are more easily reproducible.

One also feels that this method more fully reveals the condition of the thyroid in regard to its peculiar secretory function.

Since completing these studies, a well sodium iodide crystal counter* has improved the wet sample counting geometry so much that the net counting rates are three times as large. It was considered advisable to use this gain partly for increasing statistical significance, not reducing the dose given to the lowest level (10-20 $\mu$c) that will permit counting. With 40 $\mu$c doses using two gram samples the red cell:plasma activity ratio at seventy-two hours can now be measured with presumptive probable error of less than 2 per cent if the samples and background are counted for about half an hour each. This seems to make the method more available for general clinical use.

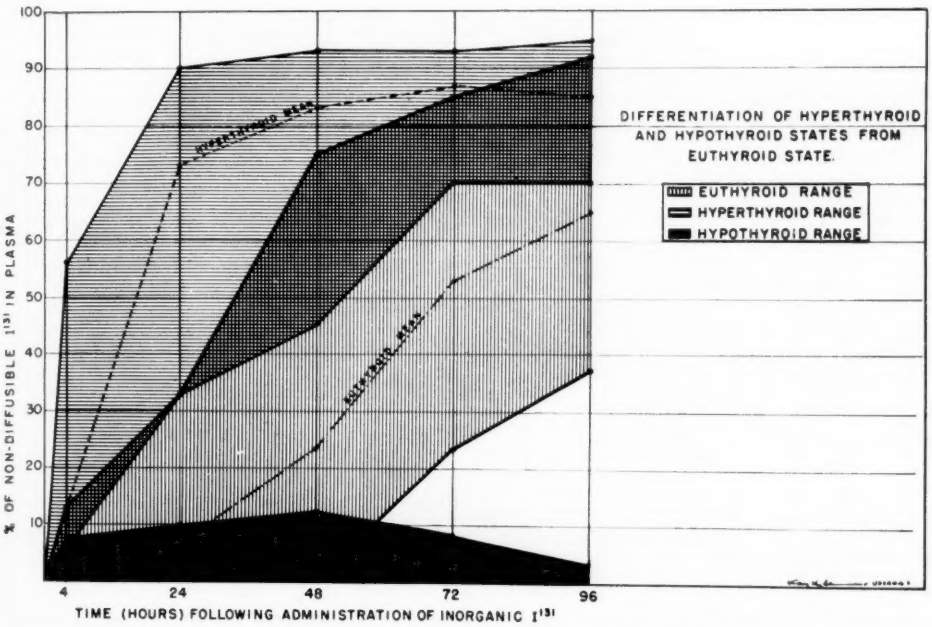


Fig. 1.—The increase of the nondiffusible fraction of $I^{131}$ activity in plasma.

## SUMMARY

1. A blood method is described which uses the determination of the red cell:plasma $I^{131}$ ratio for calculating the rate of formation of nondiffusible (organic) fraction of $I^{131}$ in plasma under varied thyroid states following the oral administration of inorganic $I^{131}$.

2. Maximum differentiation of apparent hyperthyroid and euthyroid states was observed at twenty-four hours, whereas maximum differentiation between the hypothyroid and euthyroid states was not observed until seventy-two hours following the ingestion of inorganic $I^{131}$.

*Acknowledgments.*—The author is indebted to Dr. William A. Reilly, Director of this Unit for the management of the clinical aspects of this work, and grateful acknowledgment is also made to Mr. James N. Castle for his technical assistance.

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# RADIOACTIVE IODINE-LABELED FAT

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A METHOD of labeling fat with radioactive iodine has been devised for the purpose of studying changes in the absorption and distribution of fat in the body. The stable isotope, deuterium[1] and the radioisotopes of carbon[2-4] have been used to study the metabolism of fat. Radioactive iodine ($I^{131}$) is particularly useful in labeling fat because it forms a chemically stable compound with unsaturated fatty acids, has a convenient half-life, and may be measured easily.

Methods previously described for the iodination of chemical compounds with radioactive iodine vary depending upon the objective, but have certain limitations. These methods employ (1) crystalline iodine[5, 6] ($I^{127}$ with $I^{131}$), (2) substitution of radioactive iodine for stable iodine,[7, 8] and (3) iodine monochloride[9-11] (Wijs's solution). Wijs's and Hubl's solutions contribute not only stable iodide but an excess of chloride. In this case atoms of chlorine, stable iodine, and radioactive iodine compete with each other for addition to the double bond of the fatty acid. In all three modifications gross amounts of iodine are necessary, and hence the products have low specific activity since they contain large amounts of stable iodine as well as radioactive iodine.

After the fat has been labeled with radioactive iodine, it is essential that any of the unreacted isotope, or that which is adsorbed, be completely removed so that all iodine which remains is chemically bound to the fat. Methods using silver precipitation and extraction with reducing solutions have been useful in removing unreacted radioactive iodine.

In the method to be described, the unsaturated position in oleic acid has been labeled with radioactive iodine in a carrier-free system. The unreacted radioactive iodine is then removed from the product.

## METHOD OF CHEMICAL PREPARATION OF LABELED FATTY ACID

A solution of one to two millicuries of carrier-free sodium iodide ($I^{131}$) dissolved in one-half milliliter of distilled water is placed in a separatory funnel, and 100 ml. of ethyl ether (anhydrous) is stratified over it. Three to five drops of concentrated nitric acid is added to this closed system. It was desirable to use an oxidizing medium sufficiently strong to convert iodide to iodine, but sufficiently weak to avoid undesirable change in the fat. Approximately 25 ml. of oleic acid is dissolved in the ether phase. Initially the mixture is agitated and then allowed to stand overnight. The ether layer containing the fat is then washed four times with solutions of 10 per cent sodium thiosulfate, then four times with 10 per cent potassium iodide, and finally twice with water. These multiple extractions re-

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move the unreacted or absorbed radioactive iodine. The water extractions contain negligible amounts of radioactive iodine; therefore all the iodine is in chemical combination with the fat. The ether can then be removed and the labeled fat standardized against a radioactive iodine standard. The final product contains 40 to 50 per cent of the original radioactivity used.

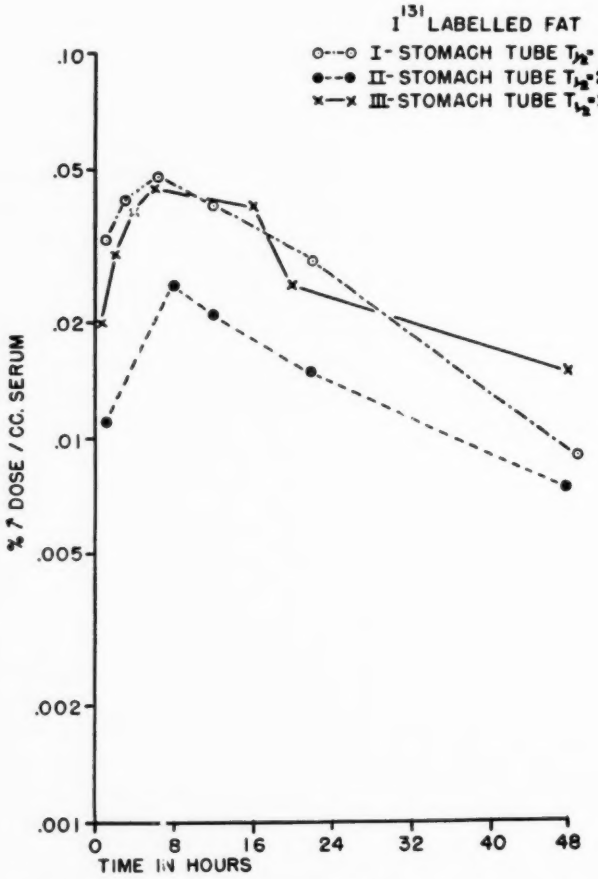


Fig. 1.—Serum radioactivity curves illustrating the percentage uptake of labeled fat ($I^{131}$) administered per cubic centimeter of serum.

## EXPERIMENTAL METHODS IN ANIMALS

The stability of the iodine linkage and the distribution of the labeled fat were studied in rabbits. The animals were maintained on a diet of Purina rabbit chow before and during the experiments. Three rabbits weighing between 2 and 3 kilograms were given labeled oleic acid (approximately 2 ml.) containing 150 microcuries of iodine$^{131}$ by stomach tube. Tween was given to increase the absorption of the fatty acid. Control observations were made by giving three rabbits of similar weight doses of radioactive sodium iodide equivalent to that contained in the oleic acid. Blood was drawn from the animals at frequent intervals to determine the rate of disappearance of the radioactivity from the blood, and at the conclusion of each experiment samples of tissues were obtained to observe the deposition of the isotope in the labeled fat. Determinations of the radioactivity were made on duplicate

samples of dried serum and solutions containing the digested tissue. The results have been expressed as the per cent of the administered dose per milliliter of serum or per gram of tissue.

## RESULTS

The distribution of the radioactivity in the blood of the two groups of animals differed. Fig. 1 illustrates the disappearance of the radioactivity from the blood of animals administered labeled oleic acid. In these animals the radioactivity reached a maximum in six hours and remained high for an additional four hours. The half-life of the labeled oleic acid in the blood was sixteen

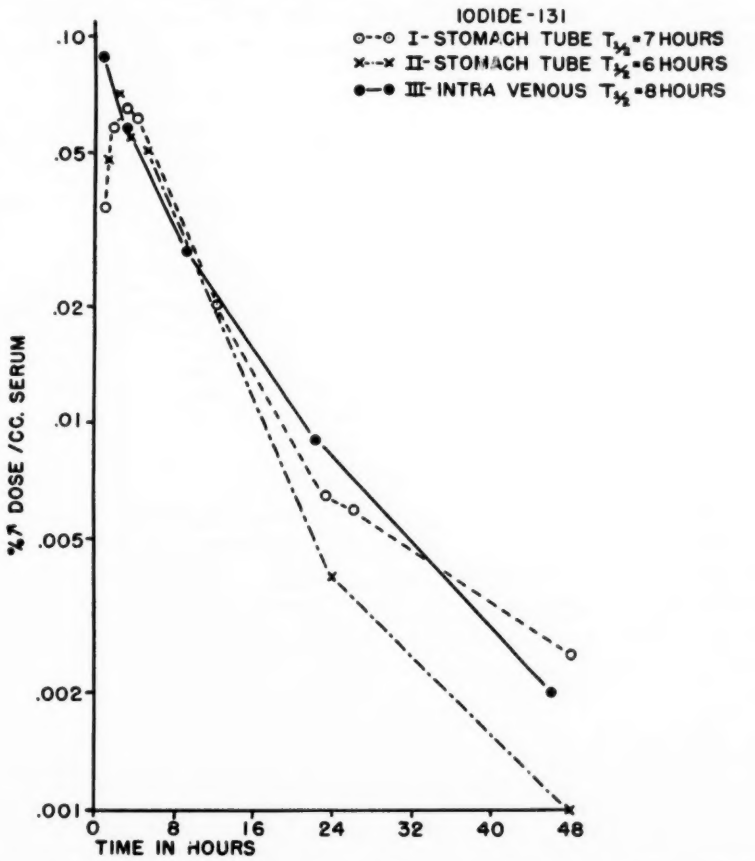


Fig. 2.—Serum radioactivity curves illustrating the percentage uptake of radioactive iodine ($I^{131}$) administered per cubic centimeter of serum.

to twenty-two hours. These curves contrast sharply with curves derived from animals given inorganic iodide$^{131}$ (Fig. 2). One animal was given iodide$^{131}$ by the intravenous route; the rate of disappearance of radioactivity from the blood of this animal was similar to that of animals administered the inorganic iodide through a tube into the stomach. In the animals receiving the inorganic iodide there was a rapid rise in the radioactivity of the serum, reaching a peak at about two hours. In these animals the half-life of the radioactivity in the

serum was six to eight hours. The maximum concentration of serum radioactivity of animals administered inorganic iodide$^{131}$ was about twice as great as in animals given labeled oleic acid.

The distribution of radioactivity of the liver and the fat depots differed in the two groups of animals, as is shown in Tables I and II.

Tables I and II illustrate the per cent of administered dose per gram of tissue found in the liver and the omental and perirenal fat depots at forty-eight hours. In the animals given inorganic iodide the accumulation in the tissues studied was negligible. In contrast animals given the labeled fat preparation possessed considerable radioactivity in the liver and fat depots. The high radioactivity in the liver probably represents the role that this organ plays in the utilization of fat.

TABLE I. IODINE$^{131}$ (MICROCURIES PER CENT OF DOSE PER GRAM TISSUE)

| RABBIT | I | II | III |
|---|---|---|---|
| Liver | 0.0009 | 0.0006 | 0.0010 |
| Omental fat | 0.0005 | 0.0002 | 0.0002 |
| Perirenal fat | 0.0004 | 0.0001 | 0.0003 |

TABLE II. IODINE-LABELED FAT (MICROCURIES PER CENT OF DOSE PER GRAM TISSUE)

| RABBIT | I | II | III |
|---|---|---|---|
| Liver | 0.0089 | 0.0060 | 0.0061 |
| Omental fat | 0.0042 | 0.0025 | 0.0039 |
| Perirenal fat | 0.0021 | 0.0010 | 0.0019 |

## SUMMARY

A method of labeling fat with radioactive iodine has been described. In biological experiments the behavior and distribution of this labeled fat has indicated that the isotope-fat bond is stable. A distinct contrast was shown between the distribution of inorganic iodide and iodine bound to fat. The results compare favorably with the data presented by others on the distribution of fat labeled with other agents.[12, 13]

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# THE BONE MARROW IN METASTATIC MALIGNANT MELANOMA

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WITH widespread utilization of diagnostic needle aspiration, there has been an increasing number of reports on metastatic tumors of the bone marrow.[4, 6, 7, 9, 10, 15] Conventionally the diagnosis of neoplasm has rested upon a study of histopathologic characteristics in stained sections of the tumor. More recently, however, under the stimulus of Papanicolaou[12] pathologists have been making tentative diagnoses of malignancy upon detailed study of single or syncytial tumor cells obtained from organ aspirates or body secretions. Although the diagnosis of tumor types on the basis of cytologic characteristics will always be difficult, and in many cases impossible, some metastatic tumor cells will exhibit features which may prove to be diagnostic for a given tumor type.

In our experience with four cases, melanoma cells metastatic in the bone marrow stain quite satisfactorily with various hematologic stains and reveal fairly distinctive cytologic characteristics. Because of the infrequency of reports on the marrow findings in metastatic malignant melanoma,[2, 5, 6, 8, 9, 14] and because no previous reports have elaborated on the supravital staining characteristics of these cells, it was thought that our observations might be of some interest.

## MATERIAL AND METHODS

We have studied the bone marrow in eight cases of malignant melanoma proved to be metastatic by biopsy or necropsy or both. In four of these eight cases tumor cells were found by marrow aspiration. Since our principal interest in this presentation is the appearance and significance of these pathologic cells, major emphasis will be centered on the positive cases. The pertinent clinical features are briefly summarized.

## CASE HISTORIES

Case 1.—A 43-year-old white man was admitted to the male medical service of the Ohio State University Hospital with many subcutaneous dark nodules, hepatomegaly, and severe normochromic anemia. A sternal marrow biopsy was performed with great difficulty and only a drop of red aspirate was obtained. On microscopic examination, this consisted almost entirely of tumor cells which were present singly and in syncytial sheets. Most cells contained minimal to large amounts of pigmented granules which appeared almost black on unstained, supravitally stained, and Wright stained preparations. The diagnosis of metastatic malignant melanoma was made and confirmed by necropsy three weeks later.

Case 2.—A 46-year-old white woman was seen at the Indiana University Medical Center with a collapsed vertebra. Seven months previously a pigmented mole in the midscapular area had been fulgurated. When it recurred it was surgically excised and histologically diagnosed as malignant melanoma with little pigment. When it again recurred

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wide surgical excision with skin grafting was accomplished. The patient then began to note paresthesias and eventually paralysis of the lower extremities with some disturbance of sphincter control. X-rays revealed collapse of the third lumbar vertebral body. X-ray therapy was initiated but was stopped because of severe nausea and vomiting. She was admitted to the hospital complaining of severe back and left shoulder pain. Sternal marrow aspiration yielded good fragments. On microscopic examination a scattering of tumor cells were found. A radiographic skeletal survey demonstrated widespread osteolytic lesions of the parietal skull, first left rib, clavicle, third lumbar vertebra, and the left iliac crest. The left iliac crest was punctured at the site of the osteolytic lesions. The bone was very fragile and the marrow cavity was entered with disturbing ease. A large quantity of aspirate was obtained with minimal effort. On microscopic examination, large numbers of vacuolated tumor cells, singly and in syncytial sheets, were seen. The great majority of these cells were nonpigmented and only rare scattered cells containing fine, sparse pigment granules could be found. Few normal marrow elements were observed in this specimen. The patient expired nine months later with widespread osteolytic lesions and severe pancytopenia.

CASE 3.—This 23-year-old white man had a pigmented skin lesion removed from the right scapular area in another hospital. (It was learned only after death of the patient that the lesion had been diagnosed as malignant melanoma by the pathologist.) Six months later the patient developed severe lumbar backache, weight loss, fever, and paresthesias of the left leg. When seen at U. S. Army Hospital, Camp Atterbury, Ind., there was icterus, a left facial palsy, and marked tenderness over the sternum and spinous processes of the lumbar vertebrae. The liver was tender, nodular, and enlarged to the umbilicus. Roentgenograms revealed metastases in both lung fields, destructive lesions of the lumbar vertebrae, and diffuse metastatic infiltration of the sacrum. Sternal puncture was performed at the site of maximum tenderness in the lower sternum and yielded little marrow material which grossly appeared acellular. Microscopic examination revealed almost complete absence of normal marrow constituents; however, many nonpigmented tumor cells, single and syncytial, as described below, were seen. The patient died nine days after admission. Necropsy revealed extensive spread of nonpigmented malignant melanoma to lungs, liver, spleen, lymph nodes, pancreas, pericardium, kidney, adrenal, and bone marrow.

CASE 4.—This 24-year-old white man, seen at U. S. Army Hospital, Camp Atterbury, Ind., had noted a small pigmented skin lesion at the left frontal region of the hair line for about two years. He presented himself for medical attention after irritation by a helmet had produced further growth and slight bleeding. Satellite lesions were noted around the tumor. Excision of a left postauricular lymph node revealed malignant melanoma with lymphatic metastases. Many generalized black skin lesions developed within a few months. Roentgenograms demonstrated lymphangitic pulmonary metastases and destructive lesions of the cervical vertebrae. A sternal marrow biopsy performed at the site of maximum tenderness near the fourth costochondral junction two days before death yielded scanty red aspirate. Microscopic examination revealed diffuse infiltration with nonpigmented tumor cells as described below. No normal marrow cells were seen. On necropsy there were extensive metastases to brain, lung, liver, spleen, skin, lymph nodes, and bone marrow.

Three additional patients with proved lymph node metastases but no evidence of osseous involvement were examined by bone marrow aspiration. Neither tumor cells nor pigmented reticuloendothelial cells could be recovered in any of these instances. Puncture was attempted in an additional patient at the site of a radiographically proved metastasis in the iliac crest. No aspirate was obtained, and subsequent sternal puncture was negative for tumor cells. The bone marrow preparations of the four patients without demonstrable tumor cells revealed increased plasma cells, and usually an increase in

both erythrocytic and granulocytic precursors. These features were nonspecific and were frequently seen in marrow aspirates of patients with a variety of metastatic tumors.

### CYTOLOGIC FEATURES OF MALIGNANT MELANOMA CELLS

Except for the variable presence of pigment, the cells from our four positive cases had a similar appearance and compare well with the cytologic characteristics reported by other authors. However, no spindle-shaped cells[5, 8] were seen.

We have examined the appearance of these cells with Wright's stain and with supravital stain employing neutral red and Janus green. All criteria for the diagnosis of tumor cells in the bone marrow were encountered. Many

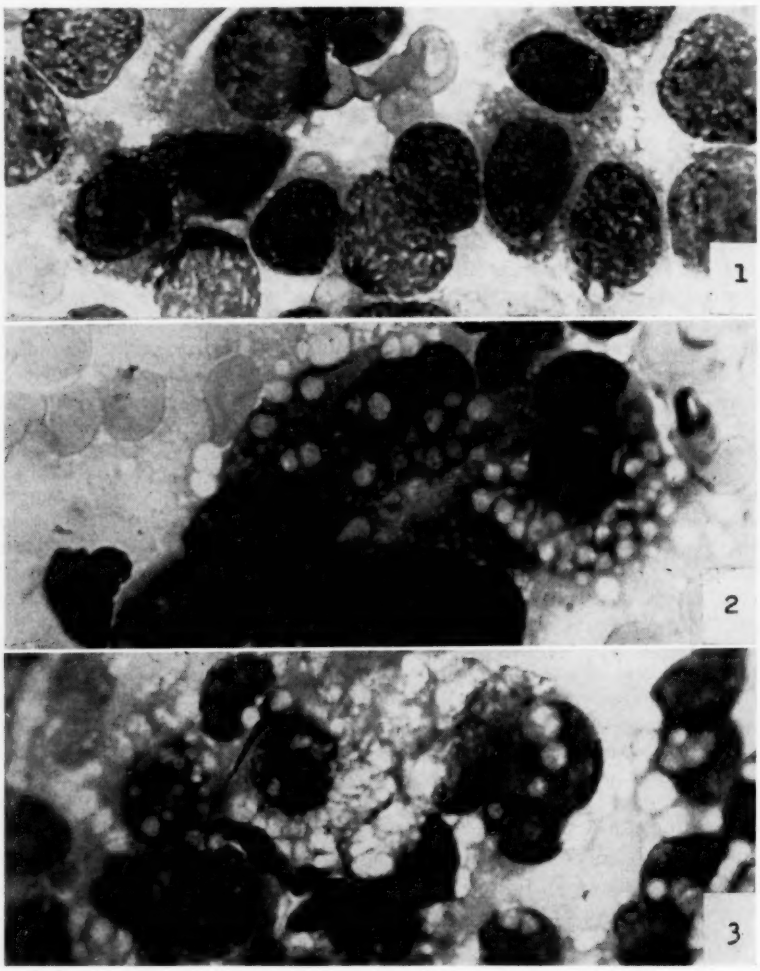


Fig. 1.—Syncytium of nonpigmented malignant melanoma cells in bone marrow. (×1000.) All photomicrographs were obtained from Wright's stained preparations.

Figs. 2 and 3.—Syncytium of malignant melanoma cells containing large vacuoles in cytoplasm. (×1000.)

isolated round to oval cells as well as cell clumps were observed (Fig. 1). Nuclear structure was bizarre, and many multinucleated cells were found (Figs. 4 and 5). Mitoses and occasional amitotic divisions were seen. The nuclei were usually large and frequently contained one to three nucleoli. The cytoplasmic border often was indistinct. Significant cytoplasmic vacuolization was noted, which in some cells was present in the form of a lattice-like arrangement of many small vacuoles in addition to larger (7 to 10 micra) vacuoles (Figs. 2, 3, and 6).

With Wright's stain the cytoplasm appeared pale blue. Nuclear chromatin was distributed in a punctate and stippled manner which sometimes almost assumed a checkerboard appearance.

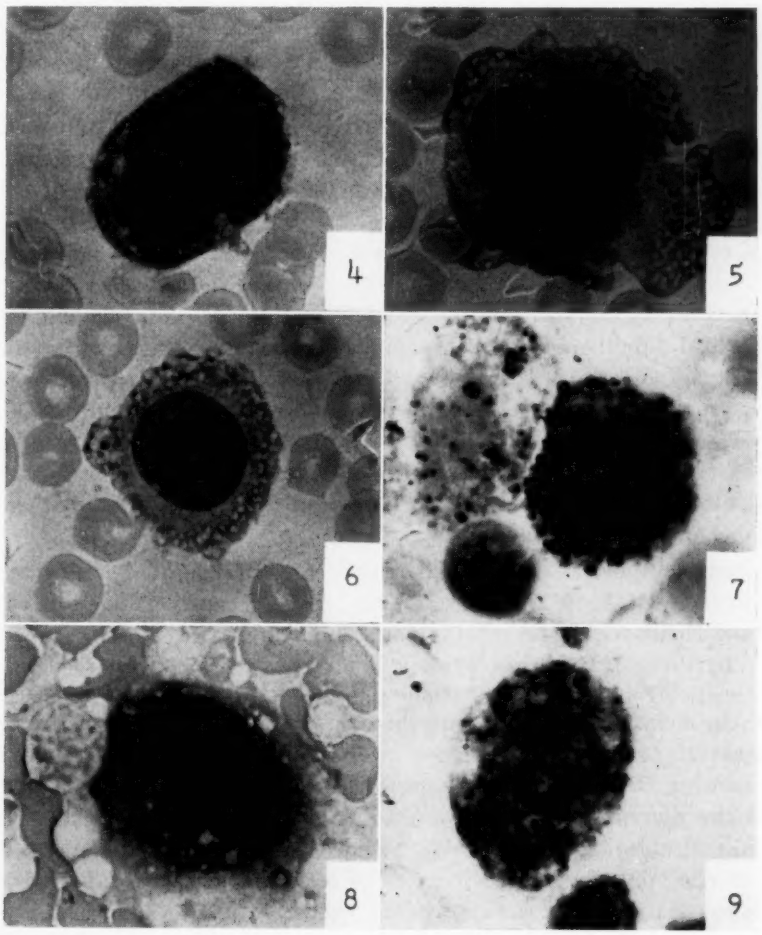


Fig. 4.—Single malignant melanoma cell with bizarre nucleus. (×1000.)

Fig. 5.—Multinucleated malignant melanoma cell. Note fine vacuolization of cytoplasm. (×1000.)

Fig. 6.—Malignant melanoma cell with fine vacuolization in cytoplasm. (×1000.)

Fig. 7.—Grossly pigmented malignant melanoma cell. Note pigment in tumor cell "Ghosts" and extracellularly. (×1000.)

Figs. 8 and 9.—Pigmented tumor cells. (×1000.)

On supravital stain (R. J. R.), the nuclei were pale and vesicular with a very fine nuclear chromatin and a well-demarcated round nuclear membrane. The cytoplasm was basophilic, containing many highly refractile vacuoles as described above. Mitochondria were few in number, small, round, and disseminated diffusely throughout the cytoplasm. In pigmented tumor cells the pigment appeared to be present in round, irregularly sized vacuoles, which on standing became tan or brown or brownish black. This coloration seemed independent of dye employed and appeared on unstained slides and coverslip preparations. Pigment could also be seen extracellularly. It was interesting that typical tumor cells were observed to phagocytize pigment. They could be contrasted (in Case 1) with typical single oval-nucleated phagocytic macrophages in the same preparation and field. Typical pigmented tumor cells are shown in Figs. 7 to 9 (Wright's stain).

## DISCUSSION

Our experience with that of others[9] bears out that a greater number of positive yields for metastatic cells will be obtained if the following three procedures are carried out. (1) Gentle systematic palpation and percussion of bones will at times elicit sharply localized tenderness. Introduction of the needle at the point of maximum tenderness is more likely to set the tip in a focus of metastatic tumor cells. For instance, sternal puncture in a case of suspected malignancy is not necessarily done at the usual location (between the second and third costochondral junction), but at the site of maximum tenderness on palpation (as in Cases 3 and 4). (2) Radiographic skeletal surveys may reveal localized osteolytic or osteoblastic lesions where ample numbers of tumor cells may be obtained. (3) At least six films of a given aspirate should carefully be examined before a bone marrow biopsy is reported negative for tumor cells. Special attention should be paid to the edges of the preparations where syncytial sheets of tumor cells tend to aggregate.

Various authors have reported single cases of malignant melanoma diagnosed by bone marrow puncture.[2, 6, 9, 14] Very recently two additional cases were published[8] which brings the total number of previously reported cases to 10. In three instances a grossly black aspirate was obtained.[6, 8, 14] Dubois-Ferriere[5] reports on the bone marrow findings of four patients with metastatic melanoma. In two of these he observed melanin pigment in the cytoplasm of marrow macrophages in the absence of actual tumor cells. In the remaining two patients both tumor cells and pigmented macrophages were seen in the marrow. Case 1 of our series showed similar findings. It is evident that circulating melanin will be filtered by the reticuloendothelial elements of the bone marrow giving rise to macrophages with ingested pigment. For diagnostic purposes such a finding would be just as helpful for the diagnosis of malignant melanoma as the actual demonstration of tumor cells since generalized dissemination of melanin by the blood stream always points to metastatic malignant melanoma. However, other forms of pigmentation may mimic the appearance of melanin. Macrophage pigmentation in malaria may be dark black and possibly could cause confusion.[11] Though such cells so far

have been demonstrated only in malarial spleen puncture material, they conceivably could be recovered from bone marrow aspirates. It is generally agreed, however, that malarial pigment is hematin-like and not melanin.[3] Hemosiderin in rare cases[1, 8, 13] may appear as blackish pigment within reticuloendothelial cells. Ordinarily, hemosiderin granules should not cause confusion since they usually stain in varying shades of green to blue by Wright's and Giemsa's stains. In unstained preparations hemosiderin appears tobacco yellow to brown. Definite characterization may be accomplished by the Prussian blue technique. Moeschlin[11] describes azure-blue, fine, stippled pigmentation in macrophages of bone marrow and spleen. The nature of the pigment is yet unknown. This has been observed in the marrow of a patient with possible chronic malaria and in splenic puncture aspirate of another patient with idiopathic splenomegaly not associated with hemolytic anemia.

Practically an absolute diagnosis of malignant melanoma can be made by bone marrow aspiration if the aspirate is grossly black, or if black granules are found in the cytoplasm of the characteristic tumor cells. In occasional cases extracellular pigment may be present also[14] (Fig. 7). It is our feeling that the cytologic appearance of these cells, even in the absence of pigmentation, is fairly characteristic. These cells can be distinguished easily from the mucous containing metastatic tumor cells seen in many instances of bronchogenic carcinoma, adenocarcinoma of the stomach, and carcinoma of the colon. Hypernephroma tumor cells often resemble early plasma cells which may give confusion with multiple myeloma, but should cause no difficulty in cytologic differentiation of malignant melanoma. Prostatic and breast carcinoma tumor cells usually are quite undifferentiated. The marked vacuolization of some malignant melanoma cells may give superficial difficulties in differentiation from the "foam cells" of Hand-Schüller-Christian and Niemann-Pick disease. Any similarity is quite spurious, since the cytoplasm of true "foam cells" is very large with complete replacement of normal cytoplasmic structure by the pathologically stored lipid. It is our impression that further experience with different tumor cells will help to delineate criteria making definite diagnosis feasible in many instances.

The majority of malignant melanomas metastasize first to lymph nodes and only late in the disease is hematogenous dissemination evident. Though it is realized that malignant melanoma may metastasize widely, bone involvement has not attracted much attention. A recent review on the topic,[16] from the x-ray point of view, arrives at a figure of 4.2 per cent bone involvement in 989 cases collected. The authors personally studied 83 cases and demonstrated metastatic bone lesions roentgenologically in nine of their patients (10.8 per cent). Of these 83 patients only 45 had been studied by x-rays and only 15 had complete skeletal survey. No characteristic x-ray appearance of malignant melanoma bone lesions could be demonstrated. The lesions resembled those seen in other osteolytic bony tumors. In all cases which were reviewed as well as in their own series, the lesions were invariably osteolytic and there was no tendency to bony proliferation. The auxiliary value of bone marrow aspiration is not mentioned in any of these reports.

All our four cases had advanced metastatic disease and bone marrow puncture confirmed what was known clinically about these patients. For medicolegal reasons definite proof of malignant disease in a given patient is always desirable and sometimes necessary. Case 3 of our series presented such a problem on admission to the hospital which was solved by the finding of the positive bone marrow aspirate. It is apparent that demonstration of tumor cells would be of greatest clinical importance when no other evidence of metastases exists. Thus in at least two reported cases[2, 5] malignant melanoma tumor cells were seen in the bone marrow many months before metastatic spread could otherwise be demonstrated.

For these reasons bone marrow puncture and radiographic skeletal survey is advised in all cases of malignant melanoma where extensive and mutilating surgery is contemplated. Demonstration of diffusely spread metastases by these procedures will then indicate to the physician the futility of any further operative procedures and will be of great diagnostic and prognostic significance.

## SUMMARY

1. Needle biopsy of the bone marrow was performed in eight patients with metastatic malignant melanoma. Tumor cells were found in four of these patients.

2. Recent literature confirms that bone involvement in metastatic melanoma is more frequent than commonly appreciated.

3. A grossly black marrow aspirate or the presence of black pigment in tumor cells of the marrow is diagnostic of metastatic malignant melanoma.

4. Even in the absence of pigment, tumor cells of the marrow in malignant melanoma have a rather characteristic cytologic appearance which may prove to be diagnostic.

5. Marrow puncture at the point of maximal bony tenderness or at sites of radiographically proved metastases will yield a larger number of marrow aspirates with demonstrable tumor cells.

6. Bone marrow biopsy and radiographic skeletal survey to rule out osseous metastases should be performed on all patients with malignant melanoma before radical mutilating surgery is contemplated.

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# THE INFLUENCE OF ROENTGEN RAYS UPON THE DEVELOPMENT OF THE MOUSE BY LOCAL IRRADIATION OF SOME PARTS OF THE BODY

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## INTRODUCTION

NUMEROUS authors have investigated the effects of local irradiation upon the growth and development of treated parts of the mammalian body. Försterling[1-3] irradiated the limbs and head of very young dogs, rabbits, and goats with x-rays. Observations were made over a long period of time (seven and one-half months), but the animals were not investigated histologically. Furthermore, none of the old papers stated the amount of irradiation which was used. Exact methods for the measurement of irradiation and the international unit of x-ray irradiation (r) were established much later. Some authors made histologic studies of locally treated parts of mammals (Hinkel[4]; Barr, Lingley, and Gall[5]; and others), but they used comparatively old animals at ages of 30, 90, and 180 days, and observed these animals for a comparatively short period of time (two months) after treatment. Regen and Wilkins[6] made observations during an eight-month period and made some histologic slides, but they used 25-day-old rabbits and gave no interpretation of the histologic pictures, which were themselves not too clear.

The purpose of the present work was to investigate the effects of localized x-ray treatment of young mice. The head and a narrow band between the fore and hind limbs were the regions irradiated.

## MATERIAL AND METHODS

Four hundred ninety-two mice of the $C_{57}$ (black) strain were used. In the first group of 47 mice, the heads were irradiated with 3,000 r (mortality : 20). The second group included 359 animals in which a narrow band midway between the fore and hind limbs was irradiated. In this second group 11 mice were irradiated with 3,000 r (mortality : 4), 18 mice were irradiated with 3,400 r (mortality : 0) and 330 mice with 4,000 r (mortality : 182). Eighty-six mice served as untreated controls. Most of the mice of the second group (121 mice) were irradiated at the age of 1 to 2 days, and 103 were treated at the age of 3 to 4 days. Sixty-five mice were irradiated at the age of 5 to 6 days, and 70 mice received their localized radiation at ages ranging from 7 to 25 days (see Fig. 1, *A* and Fig. 3).

It was necessary to use a large number of animals because irradiation of such young nursing mice resulted in a high mortality. This was especially great when they were 1 to 2 days old, and gradually decreased with age, Fig. 3).

The conditions of irradiation were as follows: 55 kv. max., 3 Ma., no filter; the target-object distance was 12 cm. The small mice were immobilized during treatment by means of strips of adhesive tape. For irradiation of the lumbar portion of the body, animals were

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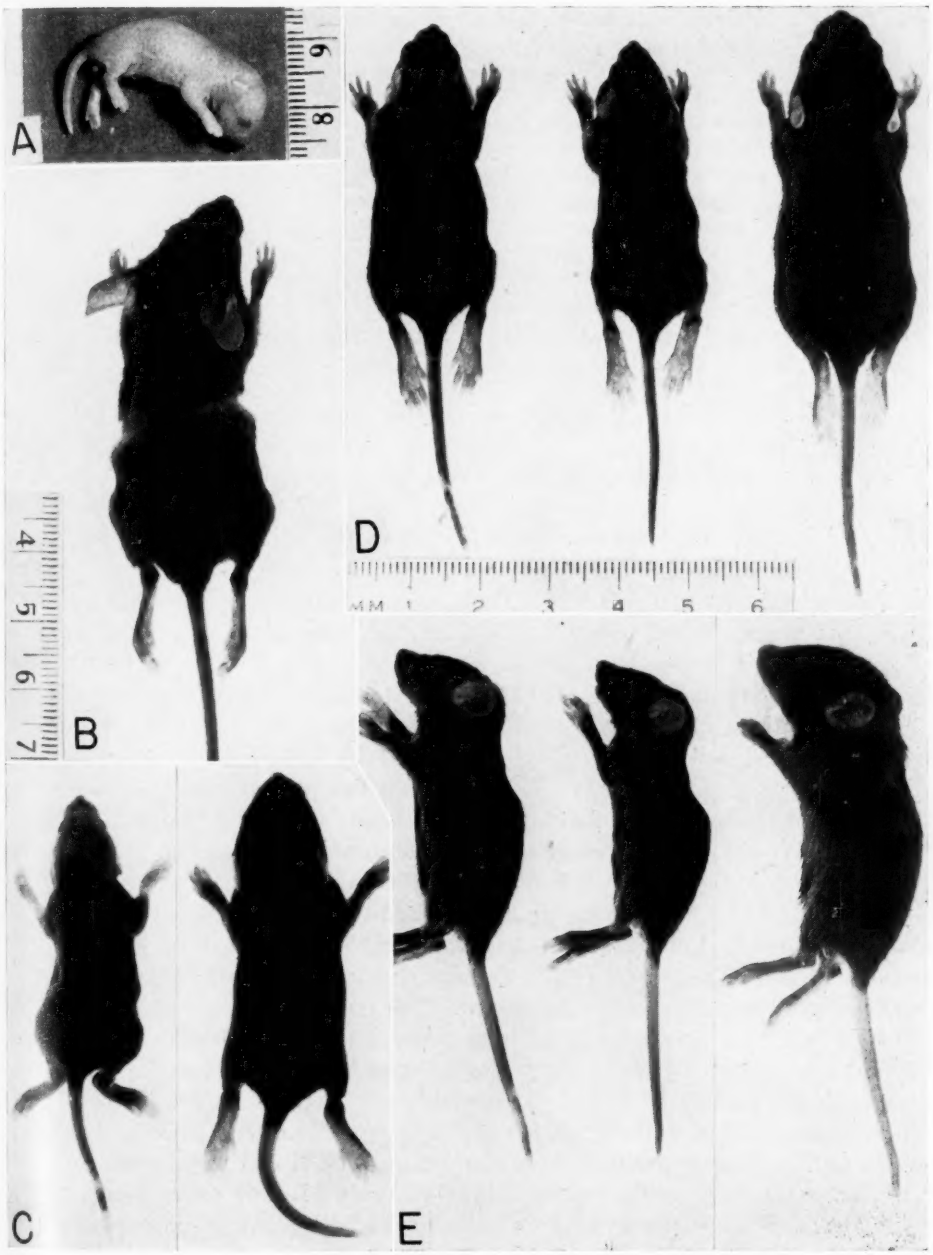


Fig. 1.—*A*, Two-day-old mouse on the day of irradiation. *B*, A mouse nineteen days after irradiation of the middle portion of the body with 3,400 r. In this mouse, there was complete paralysis of body caudal to the irradiated area. *C*, An experimental mouse (left) seven days after irradiation of the head with 3,000 r and a control mouse (right) from the same litter (dorsal aspect). *D*, Two experimental mice nine days after irradiation of the heads with 3,000 r and a control mouse (right) from the same litter (dorsal aspect). *E*, Side views of the same animals.

shielded with a 4 mm. lead cover with a localizer slit 4 mm. in width. A special cover was used during the irradiation of heads. It consisted of a lead sheet 4 mm. in thickness with a hole 3 cm. in diameter at its center, forming the opening of a well with walls of lead. Openings at the bottom of this well permitted heads to be introduced into the enclosure for irradiation. The beam of x-rays was directed vertically down into the well. Four or five mice could be treated simultaneously with this apparatus.

After irradiation, the animals were observed and photographed. In some cases, motion pictures were taken. The experimental mice were under observation for many months and, in some cases, for more than one year. Animals were fixed at various times from five days to more than one year after irradiation. The fixations were made by means of a saturated solution of bichloride of mercury with 5 per cent acetic acid and Zenker with acetic acid. The fixed material was embedded in paraffin, and sections were made at 10 microns. The sections were stained with Ehrlich's hematoxylin and eosin. In many cases, photomicrographs were made of parts of sections.

## RESULTS

*A. Some Effects of X-rays Upon the Development of the Head.*—It was found that the 3000 r dose to the region of the head is lethal for the young mouse. As early as seven days after treatment, suppression of the development of the irradiated region was evident. After irradiation of the head, we have observed not only local but general effects of irradiation as well. Not only was head growth suppressed, but there was slight but definite growth suppression of the entire body. This is evident in a comparison of such experimental animals with their controls (Fig. 1, *D* and *E*). In some of these treated animals this difference is especially noticeable in the limbs (Fig. 1, *C*). Baermann and Linsen[7] similarly observed a general effect after local irradiation of the head. These authors think that the cause of this is the influence of disintegration products which are formed in cells damaged by irradiation. According to Försterling,[1, 2] it is possible to partially inhibit the general growth of the body in dogs and rabbits by irradiating only the head. Demel[8] irradiated only the head in 4-day-old dogs and observed a clear suppression of growth of the entire body. It should be mentioned here that irradiation of the head always involves irradiation of the hypophysis, and the general inhibition of growth may be related to the effect of irradiation on this growth-regulating gland.

Nine days after treatment, a definite disturbance of brain function was manifested in disturbances of movement. The irradiated mouse fell over upon its back and made occasional convulsive movements with the limbs. After a few seconds, the animal was able to stand up, but after some minutes the same phenomenon was repeated. These paroxysms were observed in nearly all of the mice whose heads were irradiated. A similar phenomenon has been described by Demel,[8] who observed that 4-day-old dogs exhibited disturbances in walking and fell occasionally seven weeks after irradiation. In our experiments, about nine to ten days after treatment animals in the irradiated group began to die in great numbers, and the few which remained were fixed.

The paroxysms which have been described show that certain parts of the brain, or closely associated structures, suffered real damage, but no histological evidence of this was found.

More definite alterations were observed in other tissues. Nine days after treatment, endochondral ossification in head cartilage was inhibited, and the

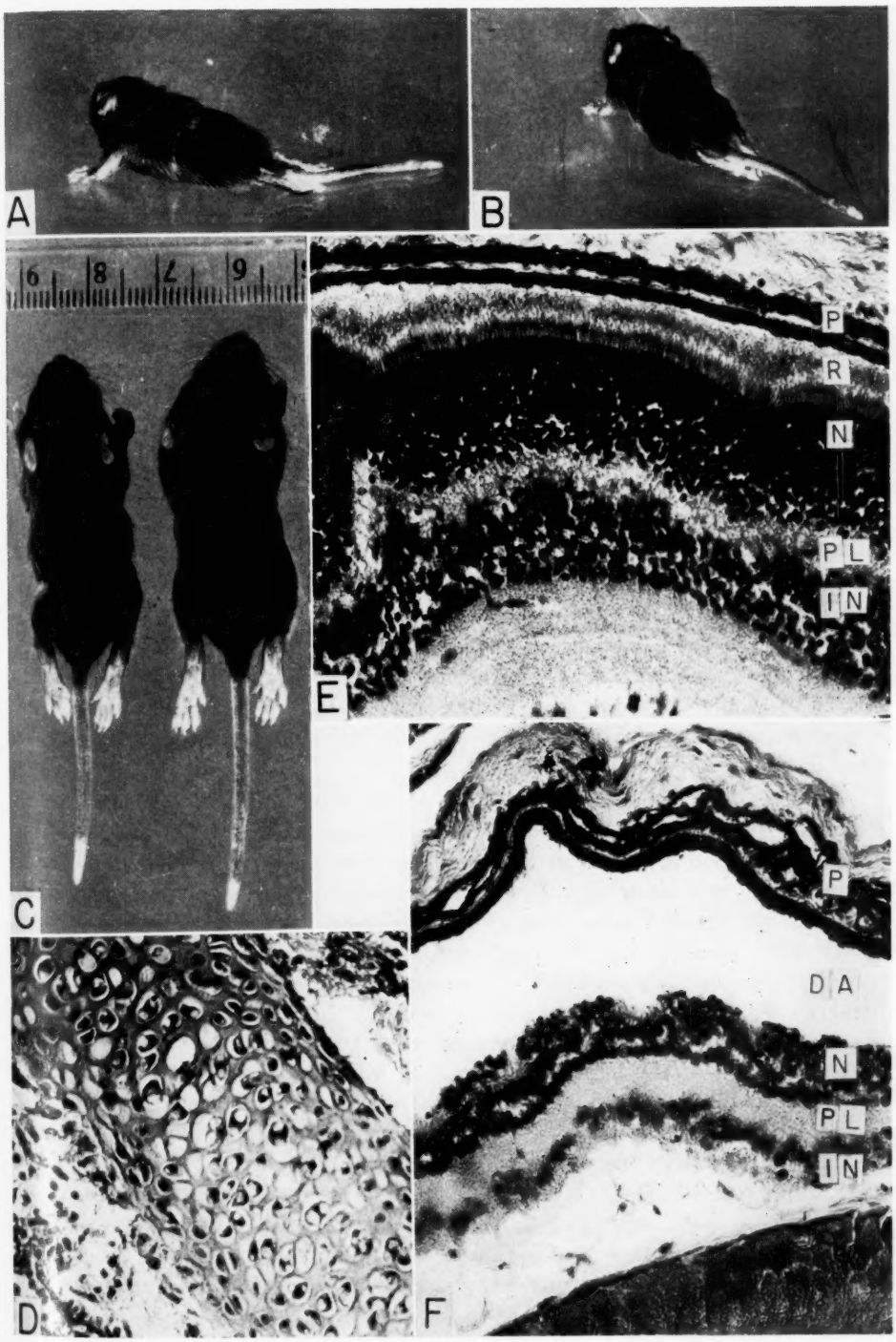


Fig. 2.—*A* and *B*, Nonnarcotized 17-day-old mouse with complete paralysis irradiated at the age of 2 days. *C*, Comparison of 16-day-old mouse with complete paralysis (left) and mouse from the same litter without paralysis (right) irradiated at age of 1 day. *D*, Cartilage in the irradiated part of the head. *E*, Sagittal section through the retina of the eye of control animal which is the same age as the animal in *F*. *P*, pigment layer; *R*, rods and cones layer; *N*, outer nuclear layer; *P.L.*, outer plexiform layer; *I.N.*, inner nuclear layer. (Photomicrograph ×280.) *F*, Sagittal section through the retina of the eye of an animal seven days after irradiation with 3,000 r. *P.*, pigment layer; *D.A.*, area in which rods and cones layer disappear; *N*, reduced outer nuclear layer; *P.L.*, outer plexiform layer; I.N., reduced inner nuclear layer. (Photomicrograph ×280.)

multiplication of cartilage cells was arrested. Cartilage with suppressed development showed some signs of abnormality. Many cells died, leaving only an empty cavity to mark their positions. The nuclei of many other cells had a pyknotic appearance, and the cytoplasm contained large vacuoles. It is evident that this tissue is undergoing a process of degeneration (Fig. 2, *D*).

Definite alterations were observed in the eyes of treated animals. Clear evidence of damage was observed only in the retinas of treated eyes in which the layer of rods and cones completely disappeared, and the outer and inner nuclear layers were greatly reduced in thickness (Fig. 2, *E* and *F*). Much has been published on the effects of x-rays on the eye, but this sensitivity of the retina has been described only in a few papers (for review of older literature see: Desjarkins,[9] Warren[10]). Rugh[11] described a disarrangement of the retinal cells in Amblystoma opacum after irradiation with 15,000 r and "significant changes in the retina" of irradiated squid.[12] In the irradiated eyes of 2-day-old rats, degeneration appears only in the "undifferentiated layers of the retina" (Spear[13]). Abelsdorff (see: Warren[10]) reported the disappearance of the layer of rods and cones after injecting into the eye an amount of thorium-X equivalent to 0.1 mg. or more of radium bromide.

The retina is probably one of the most sensitive parts of young organisms to x-ray irradiation (Kosaka[14]). Our observations about sensitivity to irradiation of the retina of young mice confirmed the data of Lorenz and Dunn[15] who showed that after irradiation of newborn mice with 400 r the layer of rods and cones disappeared in the retina.

*B. The Effect of X-ray Irradiation of the Part of the Body between the Fore and Hind Limbs.*—In some instances roentgen-paralysis was observed following irradiation of a narrow band midway between the fore and hind limbs. For example, in a litter of mice examined nineteen days after treatment with 3,400 r at the age of 4 days, some of the mice showed temporary disturbances of movements. In such mice, after a period in which normal movements were observed, there suddenly occurred a partial paralysis of the hind limbs. This paralysis disappeared after several seconds. After a time, the paralysis was observed again. This statement may be termed a "temporary paralysis" and is the first sign of the permanent paralysis which eventually follows. Thirty days after treatment, some mice in this litter showed a permanent paralysis of the hind limbs, hind part of the body, and the tail. If 2-day-old mice are irradiated, permanent paralysis may be observed as early as nineteen days after treatment with 3,400 r (Fig. 1, *B*). The paralyzed mice crept about by using the fore limbs only (Fig. 2, *A* and *B*). Paralysis was not complete in all cases. In some animals, only one hind limb was paralyzed; in others the paralysis was incomplete, and occasional movements of limbs or tail could be observed.

It is noteworthy that only some mice were paralyzed, while the others had normal movements and sensitivity. A possible explanation of this is that paralysis was observed only in animals which were irradiated exactly in the region of the motor neurons controlling the hind limbs. The photographs of treated mice with paralysis and without paralysis (Fig. 2, *C*) show that all

mice without paralysis were irradiated in a more rostral or in a more caudal region than the mice which were paralyzed completely. It is interesting to note that paralysis, when it occurred, was always observed fifteen to thirty days after irradiation.

Histologic examination showed that, in most cases, the appearance of the spinal cord of the irradiated mouse with complete paralysis is normal and cannot be distinguished from the spinal cord of a control mouse. Obviously, the damage occurs only in some cells of the spinal cord, and such damage is very difficult to establish by usual histologic examination.

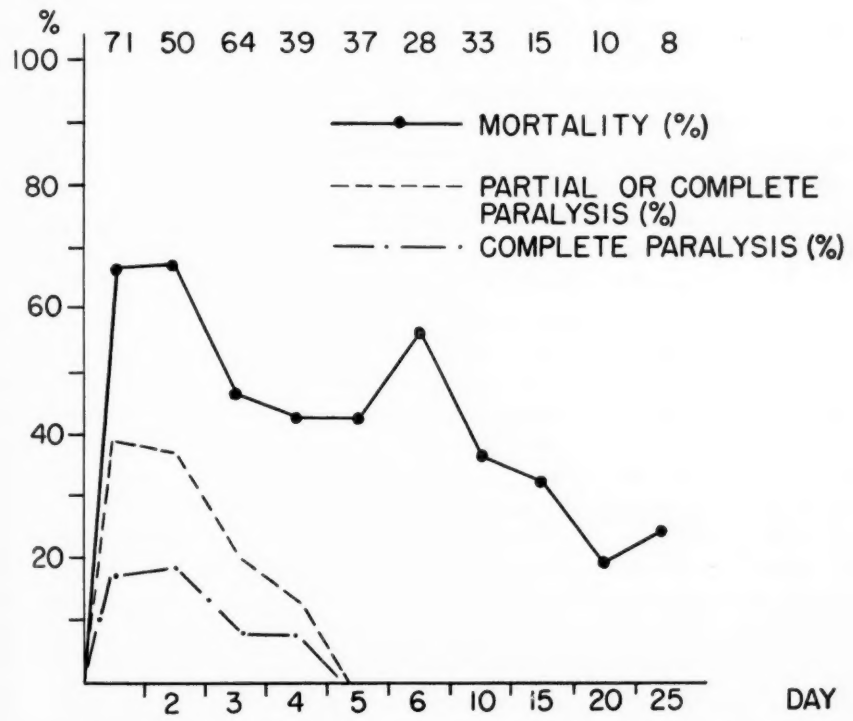


Fig. 3.—Comparison of effects of local roentgen irradiation of part of the bodies (midway between the fore and hind limbs) of mice of various ages. Figures above are numbers of irradiated animals of one age. Figures at abscissae are ages of animals in days. Ordinates—per cent of animals.

Even in the cases where the damage of the spinal cord is evident it is difficult to tell what is the primary cause of this destruction. It is possible that the damage to the cord is secondary to irradiation damage of blood vessels or skeleton. In the x-ray picture of an irradiated mouse with complete paralysis, the treated region was easily distinguished because the vertebral column in this region was especially thin (obviously a result of growth suppression). The pronounced curvature of the vertebral column in the treated region was evidently a result of the weakness of the affected portion of the column and its inability to withstand muscular forces exerted upon it. It is very possible in

this case that primary damage was observed in the vertebral column and that the destruction of the spinal cord is secondary to the suppression of growth and partial resorption of the vertebral column.

It is noteworthy that in some animals the complete paralysis caudal to the irradiated regions was observed in spite of the fact that the irradiation was not made in the lumbar region but in the chest region (Fig. 1, *B*). Obviously, in these cases, the complete interruption of the spinal cord in the irradiated region provokes secondary changes which involve the function of the motor neurons in the lumbar region and, as a result of this, complete paralysis occurs.

It is interesting that in animals with complete destruction of the spinal cord in the irradiated region, the cord in the untreated region is normal in appearance. It was found that the caudal part of the spinal cord, although isolated for a long period of time from the more cranial parts of the central nervous system by the damaged area of the cord, was alive and had a normal appearance. In spite of this, changes not easily detected must have occurred. The same observation was described in the axolotl (amphibia, Siredon mexicanum; Brunst[16]). Here, the caudal portion of the spinal cord, completely isolated from the other parts of the central nervous system, remained alive and had a histologic structure which appeared normal.

Some of the paralyzed mice can survive for a very long period of time. For example, a paralyzed mouse was fixed for histologic investigation ten months following irradiation of the middle portion of the body with 3,400 r. Investigation disclosed that the spinal cord of this animal was completely destroyed in the treated region. It is to be emphasized that the treated animal, in spite of its paralysis and destruction of spinal cord in the treated region, was able to live so long. Obviously the digestive organs, etc., continued to function well enough to support the life of this animal. The spinal cord and vertebral column of the treated region were the only parts to show distinct damage as the result of irradiation. In addition to these deeper effects, there was noted only a temporary suppression of their growth and a change from the normal hair color to gray.

Irradiation of mice of various ages shows that paralysis is observed in some cases after roentgen irradiation of mice at the ages of 1 to 4 days, but never observed following irradiation of mice 5 to 25 days old (Fig. 3). The change in x-ray sensitivity of spinal cord occurs approximately between four and five postpartum days. Our observations are in accord with the data of other investigators who have shown that the embryonic nervous system is extremely sensitive to x-rays (Kosaka,[14] Mogilnitsky and Podljaschuk,[17] Gladstone and Colwell,[18] Scholz,[19] Rugh,[11, 12, 20, 21] and others) and that nervous systems of adult animals are very resistant, (Warren,[10] Bloom,[22] and others).

Several other authors have described some paralytic symptoms, convulsive movements, and small disturbances of locomotion in irradiated mammals (Rodet and Bertin-Sans,[23] Krukenberg,[24] Walter,[25] Brunner,[26] and Gabriel[27]). But complete paralysis as a result of roentgen irradiation in mammals was not described. Such type of paralysis was described as a result of local irradiation

of the middle portion of the body of a young axolotl (Urodele Amphibia, see Brunst[16]).

## SUMMARY

In the first group of 47 mice the heads of 1- to 2-day-old mice were irradiated with 3,000 to 4,000 r. As early as seven days after treatment, suppression of the development of the treated region was evident. Not only head development was suppressed, but there was definite growth inhibition of the entire body. A definite disturbance of brain function was manifested in disturbances of movement. No histologic evidence of this was found. In eyes of treated heads some layers of retinas (rods and cones layer) disappeared.

In the second group of 359 animals, the narrow band between the fore and hind limbs was irradiated with 3,000 to 4,000 r. A paralysis was never observed following roentgen irradiation of mice at the ages of 5 to 25 days. But after irradiation of mice 1 to 4 days old, paralysis was seen in some cases. This paralysis was observed usually in animals which were irradiated exactly in the region of the motor neurons controlling the hind limbs.

Histologic examination showed that in most cases the appearance of the spinal cord of the paralyzed mice is normal. Obviously, the damage occurs only in some cells of the spinal cord and is very difficult to detect by usual histologic examination. But in some animals complete destruction of the spinal cord in the treated region was observed. It is very possible that in some cases the primary damage was observed in the vertebral column and that the destruction of the spinal cord is secondary to the suppression of growth and partial resorption of the vertebral column.

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# THE MECHANISM OF THE EFFECT OF CHANGES IN BLOOD OSMOTIC PRESSURE ON THE CEREBROSPINAL FLUID PRESSURE

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WITH THE TECHNICAL ASSISTANCE OF DORIS LAMB, B.S., EDYTHE BARNES, B.A., JOAN JACKSON, M.S., AND WILLIAM BROSENE, B.S.§

THE net seepage of fluid into or out of the craniospinal space is rapidly induced in all men by modifying the craniospinal blood volume.[3] We are reporting that changes in the osmotic pressure of the blood also induce net seepage and modify the craniospinal blood volume and the cerebrospinal fluid pressure by a similar mechanism.

## MATERIALS AND METHODS

The materials and methods have been described.[3] Most of the patients studied had had recent head injuries (Table I). Included were all conscious patients on whom pressure-volume curves[4] were obtained during the months of May and June, 1951.

## OBSERVATIONS

When a patient is given water to drink, the pressure of the cerebrospinal fluid rises gradually, reaching a peak in about twenty minutes (Fig. 1). The greater the initial pressure is, the higher is the rise. The increase in pressure is so slight when the initial pressure is within the normal range, as to be exceeded frequently by other procedures that induce changes in the fluid pressure, such as coughing or straining. A basic question is whether the individuals with the greater pressor response have a pathologic mechanism for their reaction. The answer will be approached by determining whether both the rise in pressure and its disproportionality may be explained by a mechanism common to all men.

Consider the hypothesis that each man has a shift of water into the craniospinal extravascular fluid spaces as a result of the unbalancing of the hydrostatic and osmotic equilibrium across the walls of the craniospinal capillaries; that this is consequent to the dilution of the blood following the ingestion of water; and that this fluid shift is the factor inducing the pressor response. We have inferred that, whenever fluid is added to the craniospinal fluid spaces, the effect on the fluid pressure depends on the craniospinal blood volume at the time of the addition of the fluid; the smaller this initial blood volume is, the greater is the pressor response to its further diminution.[3] If this inference is accepted,

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TABLE I. THE EFFECT OF PERORAL WATER AND INTRAVENOUS HYPERTONIC GLUCOSE ON THE CEREBROSPINAL FLUID AND JUGULAR VENOUS PRESSURES AND THE "RELATIVE CRANIOSPINAL BLOOD VOLUME"

| | | INITIAL | 50% GLUCOSE 50 C.C. | | TAP WATER | | LITERS OF WATER INGESTED |
|---|---|---|---|---|---|---|---|
| Bell | t | 0 | 41 | 46 | 9 | 30 | |
| f., 39 | | | | | | | |
| cerebral | $P_F$ | 145 | 235 | 180 | 162 | 235 | |
| contusion | $P_J$ | 120 | 138 | 115 | 128 | 128 | 1.50 |
| | $V_B$–$V°_B$ | –8.3 | –15.0 | –13.0 | –9.5 | –15.6 | |
| Burl | t | 0 | 25 | 30 | 4 | 23 | |
| m., 34 | | | | | | | |
| cerebral | $P_F$ | 110 | 100 | 120 | 93 | 96 | |
| contusion | $P_J$ | 100 | 90 | 110 | 102 | 84 | 0.65 |
| Call | t | 0 | 5 | 10 | 29 | 50 | |
| m., 70 | | | | | | | |
| cerebral | $P_F$ | 135 | 127 | 160 | 127 | 162 | |
| contusion | $P_J$ | 98 | 83 | 130 | 67 | 63 | 1.60 |
| | $V_B$–$V°_B$ | –5.8 | –8.1 | –3.9 | –11.6 | –16.3 | |
| Chen | t | 0 | 43 | 48 | 32 | 41 | |
| m., 38 | | | | | | | |
| cerebral | $P_F$ | 40 | 69 | 73 | 50 | 70 | 0.15 |
| contusion | $P_J$ | 84 | 80 | 90 | 75 | 86 | |
| | $V_B$–$V°_B$ | 4.5 | –10.8 | –8.5 | –5.0 | –8.6 | |
| Floy | t | 0 | 40 | 45 | 9 | 30 | |
| m., 47 | | | | | | | |
| cerebral | $P_F$ | 150 | 230 | 175 | 162 | 192 | 0.73 |
| contusion | $P_J$ | 130 | 155 | 135 | 132 | 139 | |
| | $V_B$–$V°_B$ | –12.0 | –18.7 | –15.5 | –13.7 | –17.2 | |
| Gard | t | 0 | 25 | 30 | 2 | 23 | |
| m., 60 | | | | | | | |
| cerebral | $P_F$ | 215 | 260 | 160 | 213 | 205 | |
| contusion | $P_J$ | 25 | 29 | 30 | 31 | 21 | 0.55 |
| | $V_B$–$V°_B$ | –13.9 | –14.7 | –12.4 | –13.8 | –13.8 | |
| Gues | t | 0 | 27 | 32 | 5 | 24 | |
| m., 47 | | | | | | | |
| cerebral | $P_F$ | 75 | 153 | 103 | 88 | 94 | |
| contusion | $P_J$ | 55 | 115 | 73 | 60 | 50 | 1.02 |
| | $V_B$–$V°_B$ | –3.5 | –6.5 | –5.3 | –4.9 | –7.6 | |
| Higg | t | 0 | 30 | 35 | | | |
| m., 25 | | | | | | | |
| cerebral | $P_F$ | 140 | 150 | 168 | | | |
| contusion | $P_J$ | 115 | 128 | 135 | | | |
| | $V_B$–$V°_B$ | –10.7 | –10.3 | –11.4 | | | |
| Hupp | t | 0 | 7 | 12 | 32 | 53 | |
| m., 68 | | | | | | | |
| cerebral | $P_F$ | 80 | 110 | 92 | 73 | 101 | 1.00 |
| contusion | $P_J$ | 50 | 61 | 43 | 35 | 29 | |
| | $V_B$–$V°_B$ | –16.5 | –19.9 | –19.9 | –16.9 | –21.8 | |
| Jame | t | 0 | 48 | 53 | | | |
| f., 45 | | | | | | | |
| metastatic | $P_F$ | 250 | 350 | 245 | | | |
| neoplasm | $P_J$ | 50 | 66 | 90 | | | |
| to skull | | | | | | | |
| | $V_B$–$V°_B$ | –25.4 | –27.5 | –23.8 | | | |

TABLE I—CONTINUED

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| John m., 62 | t | 0 | 48 | 53 | 25 | 46 | |
| cerebral | $P_F$ | 140 | 193 | 180 | 170 | 188 | |
| contusion | $P_J$ | 120 | 130 | 153 | 123 | 131 | 0.35 |
| | $V_B$–$V°_B$ | 0 | –7.2 | –1.7 | –5.5 | –6.2 | |
| Jone f., 38 | t | 0 | | | 48 | 69 | |
| cerebral | $P_F$ | 100 | | | 122 | 138 | 0.75 |
| concussion | $P_J$ | 90 | | | 87 | 101 | |
| | $V_B$–$V°_B$ | –15.2 | | | –20.4 | –20.6 | |
| Kize f., 51 | t | 0 | 38 | 43 | 3 | 24 | |
| cerebral | $P_F$ | 20 | 74 | 112 | 16 | 57 | 0.80 |
| contusion | $P_J$ | 90 | 125 | 155 | 89 | 99 | |
| | $V_B$–$V°_B$ | 15.2 | 9.2 | 5.6 | 14.7 | 4.9 | |
| McCo m., 30 | t | 0 | 95 | 100 | 19 | 40 | |
| subdural | $P_F$ | 300 | 435 | 340 | 320 | 463 | |
| hematoma and cerebral contusion | $P_J$ | 125 | 170 | 150 | 146 | 123 | 1.50 |
| | $V_B$–$V°_B$ | –18.2 | –20.3 | –18.7 | –18.0 | –21.4 | |
| Polk, m., 33 | t | 0 | | | 92 | 113 | |
| gunshot | $P_F$ | 100 | | | 205 | 205 | |
| wound of spinal cord | $P_J$ | 85 | | | 148 | 143 | 1.00 |
| | $V_B$–$V°_B$ | –1.8 | | | –8.4 | –8.6 | |
| Spru m., 80 | t | 0 | 58 | 63 | | | |
| cerebral | $P_F$ | 100 | 140 | 107 | | | |
| thrombosis | $P_J$ | 70 | 63 | 73 | | | |
| | $V_B$–$V°_B$ | –13.6 | –18.6 | –14.2 | | | |
| Sten m., 67 | t | 0 | 47 | 52 | 26 | 45 | |
| cerebral | $P_F$ | 60 | 100 | 92 | 72 | 72 | |
| contusion | $P_J$ | 45 | 50 | 65 | 39 | 39 | 0.18 |
| | $V_B$–$V°_B$ | –10.1 | –17.0 | –13.4 | –14.6 | –14.8 | |
| Stew m., 27 | t | 0 | 20 | 25 | 31 | 49 | |
| cerebral | $P_F$ | 225 | 205 | 210 | 189 | 250 | 0.55 |
| contusion | $P_J$ | 140 | 125 | 150 | 143 | 120 | |
| | $V_B$–$V°_B$ | –16.6 | –16.3 | –15.0 | –13.7 | –18.5 | |
| Tayl f., 30 | t | 0 | 39 | 44 | 11 | 29 | |
| cerebral | $P_F$ | 40 | 125 | 100 | 50 | 72 | |
| contusion | $P_J$ | 50 | 90 | 85 | 60 | 80 | 0.80 |
| Wall m., 50 | t | 0 | | | 34 | 55 | |
| cerebral | $P_F$ | 50 | | | 41 | 51 | 0.45 |
| contusion | $P_J$ | 80 | | | 70 | 82 | |
| | $V_B$–$V°_B$ | 14.3 | | | 14.4 | 14.3 | |

The sex, age in years, and diagnosis are included in the patient column. Zero time (t) is that time when the pressures became stabilized after the needling. The later times indicate the elapsed time in minutes at the start of each procedure and at an arbitrary time afterwards. $P_F$ refers to the cerebrospinal fluid pressure and $P_J$ to the internal jugular venous pressure. These pressures are measured in millimeters of water and are referred to the left atrial level. The volume $V_B$–$V_B°$ is the "craniospinal blood volume" in milliliters referred to this blood volume, $V_B°$ at the point of inflection of the pressure-volume curve.

the change in craniospinal blood volume in milliliters that is associated with a particular change in cerebrospinal fluid pressure in millimeters of water may be determined empirically for any man.[4] Therefore, we may express the increase in pressure that follows the ingestion of water in terms of milliliters of water added to the craniospinal extravascular fluid compartments, since this is the negative of the decrease in craniospinal blood volume associated with the observed rise in pressure. This is the first step in the solution of the problem; we may infer by this technique how much water passes across the craniospinal capillary walls as a result of drinking water. This quantity is designated by $y$.

We may test experimentally whether the rise in pressure is due to the shift of amounts of water dependent upon the volume of water drunk by determining the craniospinal pressure-volume curve for each man and the individual's pressor response to the ingestion of water, and expressing the pressor response in terms of the negative of the computed changes in vascular volume; such data is presented in the table.

A particular form of equation is specified as a first approximation in order to make the volume change zero when no water is ingested, in order to separate into two independent components the effect of the initial pressure and other factors on the volume response, and in order to make the effect depend linearly on the variables. This enables the hypothesis which we are considering to be symbolized in such a form that it can be tested quantitatively:

$$Y = w\,(A' + B'\,P_F^\circ) \quad (1)$$

where $Y$ = expected volume of net seepage induced by the procedure, on the basis of formula (1), assumed to represent y,

$w$ = liters of water given by mouth,

$P_F^\circ$ = initial cerebrospinal fluid pressure,

$A', B'$ = constants, the regression coefficients, determined by the data,

$y = (V_B' - V_B'') = (V_B' - V_B^\circ) - (V_B'' - V_B^\circ)$,

$V_B$ = volume of craniospinal intravascular blood, and the differences in volumes indicate the craniospinal vascular volume referred to the volume at the point of inflection of the pressure-volume curve at the start of the procedure, $V_B' - V_B^\circ$, and approximately at the time of the peak response, $V_B'' - V_B^\circ$, which was taken to be twenty minutes after the fluid was ingested. These relative volumes are determined from the individual pressure-volume curves, the parameters of which are not presented.

Substitution of the data given in the table give for the regression coefficients the following values:

$$A' \pm s_A'^{*} = 5.7 \pm 1.5,\ t^{\dagger} = 3.86,\ P^{\ddagger} = .01 - .001$$
$$B' \pm s_B' = -.14 \pm .08,\ t = 1.77,\ P = 0.1$$

The regression coefficient B′ is not statistically significant; of the total variance, the 7.7 per cent accounted for by the diversity in the initial pressures is small, and a variance component of this degree would be exceeded by chance

*Standard error of the regression coefficient.
†"Student's" $t$ (1).
‡Probability of the chance occurrence of such a high regression coefficient.

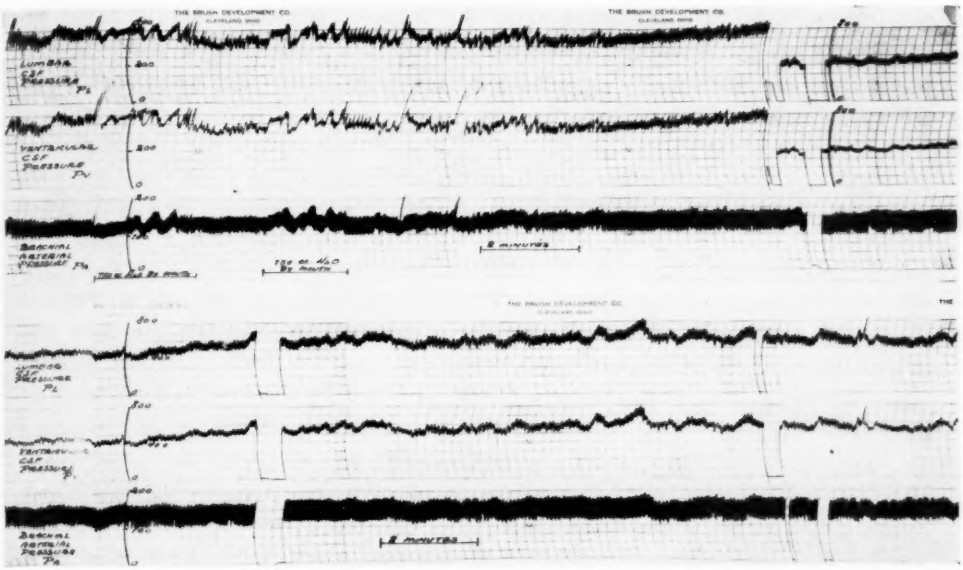


Fig. 1.—The lumbar subarachnoid fluid pressure, $P_L$, and the pressure in a lateral ventricle, $P_V$, are recorded in millimeters of water. The brachial arterial pressure, $P_A$, is recorded in millimeters of mercury. The tracings are from the record of a 30-year-old man whose cerebrospinal fluid pressure was averaging 400 millimeters of water two days after a head injury and the evacuation of a subdural hematoma. The continuously recorded pressures in the lumbar and ventricular fluid spaces and the brachial artery are shown for a period of forty minutes. All three pressures gradually rose in this case, with the lumbar and ventricular pressures doubling twenty to thirty minutes after the ingestion of one and one-half liters of water.

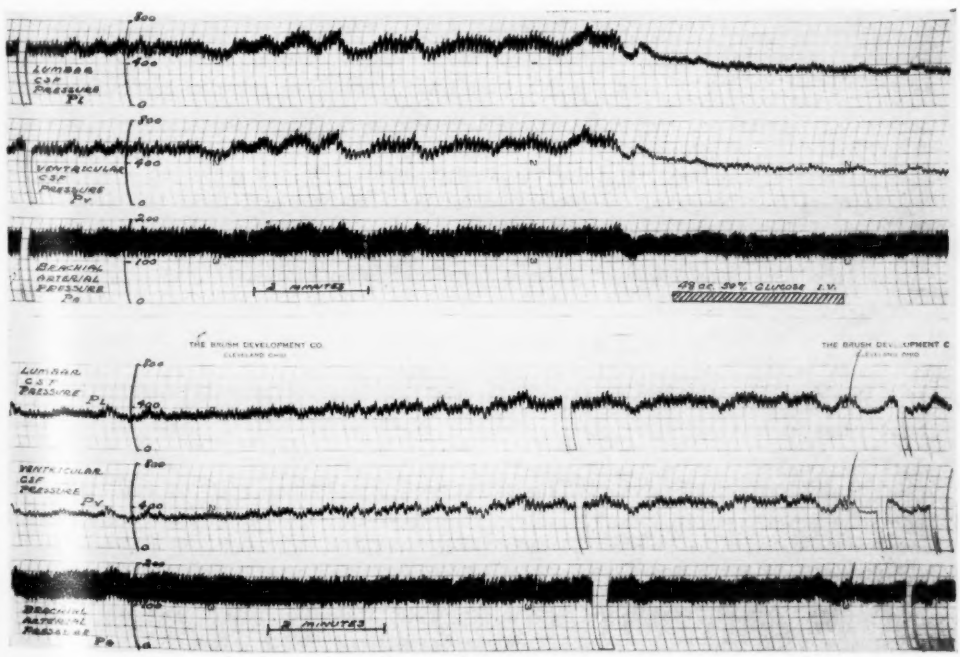


Fig. 2.—The tracing follows thirty minutes after Fig. 1. The fluid pressures dropped during and just following the administration of glucose.

not infrequently. On the other hand the volume response is significantly dependent upon the amount of water ingested. Since the effect of the initial pressure is not statistically significant we may neglect it. We are then testing the hypothesis

$$Y = A'' w \quad (2)$$

We find

$$A'' = 3.40 \pm .73, 5\% \text{ F.L.}^* = 1.80, 4.98$$

From this analysis we may conclude that when water is ingested the resultant increase in cerebrospinal fluid pressure is due to the net seepage of water into the craniospinal extravascular compartments, and the limits of this net seepage for 19 of 20 men is between two and five milliliters per liter of water drunk. This effect accounts for 60 per cent of the observed variance in the craniospinal vascular volume at the height of the effect.

The hypothesis may be subject to a more definitive test if we measure the effect of inducing an opposite change in the osmotic pressure of the blood. Suppose we induce a transient increase in the blood osmotic pressure by injecting hypertonic glucose intravenously, and estimate its effect by an analogous equation:

$$Y = A''' + B''P_F^\circ \quad (3)$$

This equation is in a simpler form than equation (1) because each man was given the same amount of glucose.

Substitution of the data in the table gives for the mean drop in craniospinal fluid volume and the dependence of the drop on the initial pressure:

$$A''' = 4.0 \pm 2.7, t = 1.47, P = .20 - .10, \text{ and}$$
$$B'' = -.05 \pm .13, t = .36, P = .80 - .70$$

Neither of these effects is statistically significant. If we make an alternative assumption that any apparent association between the extent of the drop and the initial pressure was not real, we are testing the hypothesis

$$Y = A'''' \quad (4)$$

We find

$$A'''' = 3.2 \pm 1.3, t = 2.52, P \simeq .02$$

We may conclude from these analyses that giving 50 milliliters of 50 per cent glucose intravenously did not have much of an effect, although on the average it caused about 3 milliliters of fluid to seep out of the extravascular craniospinal space (Fig. 2). This accounted for 30 per cent of the observed variance in the craniospinal vascular volume after glucose was administered. The effect was maximal five to ten minutes after the glucose was administered, but, as with water, the effect on fluid transfer was the same for each man, except for random variation, no matter what was the initial pressure or the condition from which the patient was suffering.

*5 per cent fiducial limits, which are the limits between which the regression coefficient lies, 95 per cent of the time.

## DISCUSSION

We have developed a technique of measuring indirectly the net amount of water that seeps across the walls of the craniospinal capillaries as a result of modifying the osmotic pressure of the blood. Using this method, we have found it unnecessary to assume that the difference in the pressor responses of men with normal and high initial pressures of the cerebrospinal fluid is due to an exaggeration of a normal mechanism, or to distinct processes. This last inference might be suggested from the observations of Fremont-Smith and Merritt who noted no significant effect on the cerebrospinal fluid pressure of induced changes in the blood osmotic pressure, when the fluid pressure was normal (2). The evidence presented is in favor of the hypothesis that every man has a net shift of water into or out of the craniospinal extravascular fluid spaces as a result of unbalancing the hydrostatic and osmotic equilibrium across the walls of the craniospinal capillaries, and that this fluid shift leads to the observed change in cerebrospinal fluid pressure. The transfer of water is essentially independent of either the cerebrospinal fluid pressure or the condition from which the patient is suffering.

It should be noted that the observed pressure responses could be accounted for equally well by an induced change in cerebral blood flow.[5] Since the effect can be explained without assuming that the procedures necessarily involved any change in flow, and since no changes in the systemic arterial or venous pressures or pulses occurred, we have not made this unnecessary assumption.

## CONCLUSIONS

1. A technique is described for measuring indirectly the net amount of water that seeps across the walls of the craniospinal capillaries as a result of modifying the osmotic pressure of the blood.

2. Observations of the response of the cerebrospinal fluid pressure to the ingestion of water and to the intravenous administration of hypertonic glucose are in accord with the hypothesis that every man has a net shift of water into or out of the craniospinal extravascular fluid spaces as a result of unbalancing the Starling equilibrium across the walls of the craniospinal capillaries. This shift of water leads to the observed change in fluid pressure.

3. The net transfer of water is independent of either the cerebrospinal fluid pressure or the condition from which the patient is suffering.

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# CROSS-RESISTANCE IN STAPHYLOCOCCI AND THE EFFECT OF COMBINATIONS OF ANTIBIOTICS ON RESISTANT STRAINS

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THERE have been many reports of the isolation of increasing numbers of penicillin-resistant staphylococci, particularly from patients in hospital wards.[1-9] It is of interest to the clinician to know to what antibiotics these organisms are susceptible. To determine the incidence of penicillin-resistant staphylococci in the patients admitted to Colorado General Hospital, and to what antibiotics they might be susceptible, all the staphylococci isolated by the diagnostic laboratory during a given period were tested by the disc method for susceptibility to aureomycin,† chloramphenicol,† penicillin, streptomycin, and terramycin.†

Because many of the staphylococci were resistant to two or more of the five antibiotics tested, as well as to bacitracin,** the effect of combinations of the six was determined on one penicillin-sensitive and seven penicillin-resistant strains. The penicillin-resistant strains were also resistant or only moderately sensitive to the other five antibiotics.

## PART I. DETERMINATION OF SUSCEPTIBILITY TO FIVE ANTIBIOTICS BY THE DISC METHOD.

### EXPERIMENTAL METHODS

*Organisms.*—All strains were tested for their ability to coagulate plasma. This test is generally considered to be the most closely correlated with pathogenicity. One hundred coagulase-positive and 73 coagulase-negative staphylococci were isolated from the following sources:

| Coagulase-positive | | Coagulase-negative | |
|---|---|---|---|
| Nose | 43 | Nose | 37 |
| Pus | 19 | Throat | 24 |
| Urine | 11 | Feces | 5 |
| Ear | 5 | Urine | 3 |
| Pleural fluid | 3 | Bronchus | 1 |
| Hip sinus | 3 | Urethra | 1 |
| Blood | 2 | Ear | 1 |
| Sputum | 2 | Cervix | 1 |
| Eye | 2 | | |
| Trachea | 2 | | |
| Throat | 2 | | |
| Feces | 2 | | |
| Skin | 1 | | |
| Ulcer | 1 | | |
| Cervix | 1 | | |
| Neck wound | 1 | | |

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†We are indebted to the Lederle Laboratories for kindly supplying the Aureomycin, to Parke, Davis and Co. for the chloramphenicol, to Chas. Pfizer and Co. for the terramycin, and to The Upjohn Company for the bacitracin.

*Determination of Sensitivities.*—Sensitivities were determined by the method described by Bondi and associates[10] and Spaulding.[11] Discs of blotting paper were impregnated with the antibiotics, dried, and stored over $CaCl_2$ until used. The concentrations used were as follows:

| | |
|---|---|
| Penicillin | 15 $\mu$/ml. |
| Streptomycin | 500 $\mu$g/ml. |
| Chloramphenicol | 700 $\mu$g/ml. |
| Terramycin | 500 $\mu$g/ml. |
| Aureomycin | 500 $\mu$g/ml. |
| Bacitracin | 75 $\mu$/ml. |

Blood agar plates were inoculated evenly with a swab which had been rubbed over a twenty-four-hour blood agar culture of the organism to be tested. The plates were incubated at 37° C., and the zone of inhibition around the discs measured twenty-four hours later. As a check against loss of activity by the antibiotic discs, a susceptible strain of *S. aureus* was tested concurrently with each group of strains of unknown sensitivity. At no time was any loss of activity observed. Representative strains were tested by serially diluting the antibiotics in 1 ml. of tryptose phosphate broth and inoculating with a 1-1,000 dilution of a twenty-four-hour culture. The concentration just inhibiting growth in twenty-four hours at 37° was determined, and the following criteria for resistance established.

| | CONCENTRATION REQUIRED BY THE TUBE DILUTION METHOD TO INHIBIT GROWTH *greater than* |
|---|---|
| Penicillin—zone less than 10 mm. | 1 $\mu$/ml. |
| Streptomycin—zone less than 10 mm. | 25 $\mu$g/ml. |
| Aureomycin—zone less than 8 mm. | 5 $\mu$g/ml. |
| Chloramphenicol—zone less than 10 mm. | 10 $\mu$g/ml. |
| Terramycin—zone less than 8 mm. | 5 $\mu$g/ml. |

## RESULTS AND DISCUSSION

Table I shows the percentage of coagulase-positive and coagulase-negative staphylococci that were resistant to the antibiotics studied. A high percentage (76 per cent and 82 per cent) of both types were found to be penicillin-resistant.

TABLE I. PERCENTAGE OF COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI RESISTANT TO ANTIBIOTICS STUDIED

| RESISTANT TO | 100 COAGULASE-POSITIVE STRAINS | | 73 COAGULASE-NEGATIVE STRAINS | |
|---|---|---|---|---|
| | NUMBER | PER CENT | NUMBER | PER CENT |
| Penicillin | 76 | 76 | 60 | 82 |
| Streptomycin | 37 | 37 | 27 | 37 |
| Aureomycin | 21 | 21 | 26 | 35.5 |
| Chloramphenicol | 8 | 8 | 29 | 39.7 |
| Terramycin | 29 | 29 | 25 | 34.2 |
| All antibiotics | 4 | 4 | 17 | 23.2 |

Thirty-seven per cent of the coagulase-positive organisms were resistant to streptomycin, 21 per cent to aureomycin, and 29 per cent to terramycin. Only 8 per cent were resistant to chloramphenicol, and 4 per cent to all the antibiotics. Coagulase-positive staphylococci, isolated from patients in this hospital, are more often susceptible to chloramphenicol by this method of determining sensitivity than to any of the other four antibiotics. Furthermore, one of the antibiotics would be expected to be effective for all but a small per cent of the

strains. It is fortunate that the coagulase-negative staphylococci are considered to be of less importance in disease production, since 30 per cent of those examined were resistant to all five antibiotics.

Table II points out the considerable amount of cross-resistance in the coagulase-positive strains. Nearly all of those strains resistant to streptomycin, aureomycin, chloramphenicol, and terramycin were also resistant to penicillin.

TABLE II. CROSS-RESISTANCE OF COAGULASE-POSITIVE AND NEGATIVE STAPHYLOCOCCI

| | PERCENTAGE RESISTANT TO | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PENICILLIN | | STREPTOMYCIN | | AUREOMYCIN | | CHLORAMPHENICOL | | TERRAMYCIN | |
| STRAINS | COAG + | COAG − | COAG + | COAG − | COAG + | COAG − | COAG + | COAG − | COAG + | COAG − |
| Penicillin-resistant | – | – | 44.7 | 72.0 | 27.6 | 43.3 | 10.5 | 48.3 | 38.1 | 40.0 |
| Streptomycin-resistant | 100.0 | 96.3 | – | 43.3 | 43.2 | 74.0 | 10.8 | 74.0 | 54.0 | 66.6 |
| Aureomycin-resistant | 100.0 | 100.0 | 76.1 | – | – | – | 19.0 | 96.1 | 95.2 | 76.9 |
| Chloramphenicol-resistant | 87.5 | 100.0 | 50.0 | 76.9 | 50.0 | 86.2 | – | – | 62.5 | 79.3 |
| Terramycin-resistant | 100.0 | 96.0 | 68.9 | 68.9 | 68.9 | 80.0 | 17.2 | 92.0 | – | – |

Of the penicillin-resistant strains, more were resistant to streptomycin, while a smaller percentage were resistant to terramycin, aureomycin, and chloramphenicol in that order. Spink[6] has reported finding a number of coagulase-positive staphylococci which were resistant to both penicillin and streptomycin, and Rountree reports similar findings.[12] Gocke and Finland[13] did not observe penicillin resistance in strains which developed resistance to aureomycin, chloramphenicol, or terramycin.

Several investigators have observed simultaneous development of resistance to chloramphenicol, aureomycin, and terramycin, when bacteria were exposed to any one of the three antibiotics.[14-16] Aureomycin and terramycin were more closely related in this respect than was chloramphenicol, when tested with gram-positive cocci. Table II shows 95.2 per cent of the aureomycin-resistant strains to be resistant to terramycin, but only 19 per cent to be resistant to chloramphenicol. Of the terramycin-resistant strains, 68.9 per cent were also resistant to aureomycin, but only 17.2 per cent were resistant to chloramphenicol. Aureomycin and terramycin were more likely to show cross-resistance with one another than with chloramphenicol. However, over 50 per cent of the chloramphenicol-resistant strains were resistant to both aureomycin and terramycin.

Essentially the same relationships in crossresistance were seen with the coagulase-negative staphylococci. The only noticeable difference was the nearly complete cross-resistance between all three of the antibiotics—aureomycin, chloramphenicol, and terramycin.

## PART II. THE EFFECTS OF COMBINATIONS OF ANTIBIOTICS ON RESISTANT STAPHYLOCOCCI.

### EXPERIMENTAL METHODS

*Organisms.*—Five strains of coagulase-positive staphylococci and two strains of coagulase-negative staphylococci which had been isolated from patients admitted to Colorado General Hospital were obtained in pure culture from the microbiology department's diagnostic laboratory. The eighth strain was penicillin-sensitive, and was obtained from Stanford

TABLE III. SOURCES AND SENSITIVITIES OF STAPHYLOCOCCI ON PRIMARY ISOLATION

| ORGANISM | SOURCE | COAG | MANN | ANTIBIOTIC | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | PENICILLIN | | STREPTOMYCIN | | CHLORAM-PHENICOL | | TERRAMYCIN | | AUREOMYCIN | | BACITRACIN | |
| | | | | RATING | MIC | RATING | MIC | RATING | MIC | RATING | MIC | RATING | MIC | RATING | MIC |
| Stanford | | + | + | S | .10 | MS | 5 | S | 10 | S | 1 | S | 1 | R | 5 |
| 40931 | throat | – | – | MS | 2 | R | 20,000 | R | 100 | R | 400 | R | 100 | MS | 1 |
| 40936 | urine | – | – | MS | .5 | R | 10,000 | R | 100 | R | 200 | R | 50 | MS | 1 |
| 40883 | urine | + | + | R | 25 | R | 100,000 | R | 100 | R | 400 | R | 100 | MS | 2 |
| 40902 | nose | + | + | R | 25 | R | 200,000 | R | 100 | R | 400 | R | 100 | MS | 2 |
| 41087 | nose | + | + | R | 100 | MS | 12.5 | S | 12.5 | S | 25 | S | 25 | R | 200 |
| 41157 | nose | + | + | MS | 1 | R | 100 | S | 10 | R | 100 | MS | 25 | MS | 2 |
| 41417 | nose | + | + | MS | 1 | R | 100 | S | 10 | R | 100 | MS | 25 | MS | 2 |

Coag. = coagulase reaction.
Mann = mannitol fermentation reaction.
Rating = sensitivity as determined by zone of inhibition: S = sensitive; MS = moderately sensitive; R = resistant.
MIC = minimum inhibitory concentration expressed in units per milliliter; figure represents most constant MIC of 16 determinations.

University. On primary isolation the seven organisms were resistant to one or more of the six antibiotics used, as determined by the disc sensitivity method. The source of each strain and its coagulase and mannitol fermentation reactions, as well as its sensitivities to the antibiotics, are shown in Table III.

*Determination of Sensitivities.*—The sensitivities of each of the eight strains of staphylococci were redetermined by the disc method. Preliminary experiments indicated that the zone of inhibition was not changed by using tryptose phosphate agar instead of blood agar. In order to standardize the size of the inoculum, a 1 to 10,000 dilution of a twenty-four hour broth culture of the test organism was made in tryptose phosphate agar, which was cooled to 40° to 50° C. Five milliliters of this inoculated liquid agar was poured onto a solidified tryptose phosphate agar plate. While inoculum size did not appear to influence the size of the zones with the other five antibiotics, penicillin made larger zones with some organisms when the inoculum was decreased, and these zone sizes correlated better with sensitivities determined by the tube dilution method. Six-millimeter discs of blotting paper were sterilized and impregnated with the concentrations of antibiotics given in Part I of this paper. The sizes of the zones of inhibitions were measured after twenty-four hours incubation, and the sensitivity of each strain recorded as follows:

| | Size of Zone in Millimeters | | |
|---|---|---|---|
| | Resistant | Moderately sensitive | Sensitive |
| Penicillin | <10 | 10-15 | >15 |
| Streptomycin | <10 | 10-15 | >15 |
| Chloramphenicol | <10 | 10-20 | >20 |
| Terramycin | <8 | 8-15 | >15 |
| Aureomycin | <8 | 8-15 | >15 |
| Bacitracin | <8 | 8-12 | >12 |

Minimum inhibiting concentrations (MIC) were determined by serially diluting each antibiotic in 1 ml. of tryptose phosphate broth. Each tube was inoculated with one drop of a 1 to 1,000 dilution of a six-hour culture. The tubes were read after twenty-four hours incubation at 37° C. The lowest concentration of antibiotic showing no visible growth was designated the MIC.

To determine the combined effect of two antibiotics on each strain, all of the six antibiotics were paired in every possible combination. One MIC of each of the two antibiotics being tested was mixed in a test tube. This was serially diluted to give the following concentrations:

| Tube number | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Antibiotic X | 1 MIC | ½ MIC | ¼ MIC | ⅛ MIC | 0 |
| Antibiotic Y | 1 MIC | ½ MIC | ¼ MIC | ⅛ MIC | 0 |

If there was growth in tube 1, the effect was classified as "antagonistic"; if no growth in tube 1, as "no effect"; if no growth in tube 2, as "additive"; and no growth in tubes 3 or 4, as "synergistic."

All tests were performed in duplicate, and the MIC of each antibiotic in the pair was redetermined at the same time. The method is essentially that of Romansky,[17] and admittedly misses some combinations of concentrations which might show antagonism, synergism, or an additive effect. It tests the bacteriostatic effect of the combinations and not the bactericidal, as Jawetz and his group[18] have done in their antagonism experiments. However, we believe that some useful information may be gained from such tests, and the technique is such that it can be performed in a diagnostic laboratory where time is limited.

## RESULTS AND DISCUSSION

*Determination of Sensitivity by Both Tube Dilution and Disc Method.*—The eight strains of staphylococci chosen for this study were tested for sensitivity by one of us (K.C.) when the organism was first isolated from the patient

(Part I). Sensitivity tests were again performed by the methods outlined above in the experimental procedures. Table III shows that all of those strains originally chosen for their resistance to penicillin, and one or more of the other antibiotics, were with one exception either resistant or only moderately sensitive to penicillin by both disc and serial dilution method. All were resistant, also, to one or more of the other five antibiotics tested. It is of interest to note that these eight strains of staphylococci, with but few exceptions, showed the same sensitivity by the tube dilution method as they did by the disc method using concentrations of antibiotics recommended by Bondi and associates[10] and Spaulding.[11] The only differences found between the results of the two methods were in the greater resistances reported to penicillin, when a heavy inoculum was used. This is very likely due to greater production of penicillinase by the larger inoculum, and may actually represent clinical conditions better than do the results with smaller inocula.

*Effect of Combinations of Antibiotics.*—Fifteen paired combinations of antibiotics were used against the eight strains of staphylococci, making a total of 120 tests. The results of these tests are shown in Table IV. The sixteen com-

TABLE IV. SUMMARY OF THE VARIOUS EFFECTS OF COMBINATIONS OF SIX ANTIBIOTICS ON EIGHT STRAINS OF STAPHYLOCOCCI

| COMBINATION | NO EFFECT | ADDITIVE | SYNERGISTIC | ANTAGONISTIC |
|---|---|---|---|---|
| | | strain numbers | | |
| Pen./Strepto. | 1* | 2, 7, 8 | 3, 4, 5, 6 | |
| Pen./Chloram. | 2, 3, 6 | 1, 7, 8 | 4, 5 | |
| Pen./Terra. | 3, 6, 8 | 1, 2, 7 | 4, 5 | |
| Pen./Aureo. | 1, 4, 6, 7 | | 2, 3, 5, 8 | |
| Pen./Baci. | 1, 3, 4, 7 | 2, 6, 8 | 5 | |
| Strepto./Chloram. | 2, 3, 6, 7 | 8 | 4, 5 | 1 |
| Strepto./Terra. | 1, 2, 3, 6, 7, 8 | | 4, 5 | |
| Strepto./Aureo. | 1, 6, 7 | | 2, 3, 4, 5, 8 | |
| Strepto./Baci. | 6, 7 | 8 | 1, 2, 3, 4, 5 | |
| Chloram./Terra. | 1, 3, 4, 5, 8 | 7 | 2, 6 | |
| Chloram./Aureo. | 1, 6, 7 | 2, 3, 4, 5 | 8 | |
| Chloram./Baci. | 3, 6, 8 | 1, 2, 4, 5, 7 | | |
| Terra./Aureo. | 1, 2, 3, 4, 6, 7 | 3, 8 | 5 | |
| Terra./Baci. | 2, 3, 5 | 6 | 1, 4, 7, 8 | |
| Aureo./Baci. | 1, 3, 6 | 2, 7 | 4, 5, 8 | |
| | 52 (43.3%) | 29 (24.2%) | 38 (31.7%) | 1 (0.8%) |

*Organism 1—Stanford 5—40902
2—40931 6—41087
3—40936 7—41157
4—40883 8—41417

binations displayed no effect on 43.3 per cent of the strains tested; an additive effect on 24.2 per cent; a synergistic effect on 31.7 per cent; and an antagonistic effect only once on one organism, or 0.8 per cent. These results show that it would be difficult to predict whether any combination would be effective against a particular resistant strain of staphylococci. This is in accord with the observations of others.[19-20] The effects of combinations of antibiotics were so variable that no particular pair could be chosen as one which would be likely to be either synergistic or antagonistic.

It was found, however, that penicillin and streptomycin in combination were effective, synergistically or additively, against more of the strains than any other combination. Other investigators have also noted the synergistic

and additive effects of the penicillin-streptomycin combination. Klein and Kimmelman[21] noted that low concentrations of penicillin and streptomycin were highly effective in inhibiting the multiplication of a strain of staphylococci. Kolmer[20] reported the synergistic effect of this antibiotic combination on anthrax infection of mice. Nichols[22] employed penicillin and streptomycin mixtures to inhibit the growth of strains of staphylococci and hemolytic streptococci, and noted the synergistic action of the combination. Bigger[23] demonstrated "marked synergism" with penicillin and streptomycin when used against *Escherichia coli;* and Jawetz and Gunnison[24] against *Streptococcus faecalis.* Bliss and associates[19] also reported on the synergistic activity of this combination against strains of staphylococci.

Synergistic action toward four or more of the eight strains was shown with combinations of penicillin and aureomycin, streptomycin and aureomycin, streptomycin and bacitracin, and terramycin and bacitracin. Only one example of antagonism was found in all of the combinations with all organisms. This was with streptomycin and chloramphenicol. Since additive, synergistic, or no effect was observed in nearly every case, it would appear from this in vitro evidence that the treatment of resistant staphylococci infections with combinations of antibiotics would be more likely to be helpful than harmful. Such a view seems tenable in the light of such investigations as those of Waisbren[25] and Ahern and Kirby,[26] in which excellent clinical response has followed the determination of effective bacteriostatic combinations by the tube dilution method. However, it should be pointed out that, although the concentrations needed to inhibit growth in the synergistic combinations used in this study were less than when used alone, they were not always as low as attainable blood levels. It is only when an additive or a synergistic combination is made up of concentrations attainable in the blood that the clinician could feel justified in using it with the hope of any therapeutic value.

Some strains of staphylococci were apparently more susceptible to combined antibiotic action than others. Synergistic effects occurred most often with highly resistant strains of staphylococci. Organisms 40883 and 40902, which were comparatively highly resistant to most of the antibiotics (cf. Table III, were found to be most susceptible to combined antibiotic action (synergism in 9 and 11 cases, respectively), while the Stanford strain (the "sensitive" strain of staphylococci) was least susceptible of all the strains to the action of combination of antibiotics. The concentrations necessary to inhibit growth in these synergistic combinations were often still quite high.

A study of the number of cases in which each antibiotic played a role in synergistic and additive combinations shows that streptomycin was one of the pair in eighteen synergistic combinations, but other than that no single antibiotic was involved in significantly more combinations than any other. Chloramphenicol was one of a synergistic pair less often than any of the other antibiotics.

## SUMMARY

A high percentage of penicillin-resistant coagulase-positive and coagulase-negative strains of staphylococci were found in cultures from patients in Colo-

rado General Hospital. Resistance to aureomycin, chloramphenicol, streptomycin, and terramycin was almost always accompanied by resistance to penicillin; and cross-resistance was seen between terramycin and aureomycin, but much less between these antibiotics and chloramphenicol. More strains were sensitive to chloramphenicol than to any other antibiotic.

Each of seven strains of penicillin-resistant, and one penicillin-sensitive staphylococcus differed in its susceptibility to combinations of aureomycin, bacitracin, chloramphenicol, penicillin, streptomycin, and terramycin. This indicates that each strain must be tested to determine the most effective combination for preventing growth. The combination of penicillin and streptomycin acted additively or synergistically against more strains than any other combination. The only combination to show antagonism was streptomycin and chloramphenicol, and this was shown with only one strain. Streptomycin was one of a synergistic pair more often, and chloramphenicol less often, than any of the other antibiotics. Synergistic combinations were found more often with the highly resistant strains, but the concentrations required to inhibit growth were still often very high.

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# THE EFFECT OF A BACTERIAL PYROGEN UPON THE EXPERIMENTAL CARDIOVASCULAR AND RENAL LESIONS OF ANAPHYLACTIC HYPERSENSITIVITY

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VACCINES and other pyrogenic bacterial substances have been used for many years in the treatment of various diseases[1] and, in many instances, the beneficial effect of this form of therapy is well established. There has been a recent revival of interest in the use of pyrogens for the amelioration of symptoms due to hypersensitivity. Swineford[2] reviewed the use of bacterial substances in the treatment of bronchial asthma and concluded that they had proved to be useful in the management of this disease. Randolph and Tollins[3] obtained good results in controlling the clinical manifestations of allergy with pyrogens, and suggested that the beneficial effects might be due to stimulation of the pituitary-adrenal system. Wittich[4] and Zindler[5] have also reported favorable therapeutic results in the form of symptomatic relief in allergic patients given Piromen, a *Pseudomonas* concentrate.[6] Samter, however, in a carefully controlled study,[7] could detect no difference in the response of allergic patients to Piromen and a placebo. In animal experiments, Samter found evidence of increased antibody formation in guinea pigs treated with pyrogens and similar enhancement of antibody titers by pyrogen administration has been reported by others.[8, 9]

On the basis of histologic findings in animals given pyrogens,[10, 11] as well as certain similarities between the clinical response to fever therapy and the "alarm reaction,"[12, 13] it has been postulated that pyrogens may exert a beneficial effect through adrenocorticotropic activity. Although Mitchell and Stuart[14] concluded that the leukocyte changes after pyrogen administration are dependent upon adrenal activity, Soylemezoglu and Wells[15, 16] have presented good evidence that some mechanism other than adrenal stimulation is responsible for these hematologic effects. Altschule[17] found evidence of only transient adrenal stimulation in the course of fever therapy with typhoid vaccine.

The present investigation was undertaken to determine the effect of pyrogen treatment upon the development of histologic changes in rabbits subjected to the serum sickness type of protracted anaphylactic reaction.[18] These lesions consist of arteritis, carditis, and glomerulonephritis.[19-23] Treatment with ACTH[24, 25] or cortisone[26, 27] has been shown to inhibit strikingly the development of these lesions. If pyrogen administration results in some ACTH-like action, one might expect a lessened tendency to develop these histologic changes.

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## MATERIALS AND METHODS

Sixty white male rabbits averaging 2 kilograms were used. Each animal was given normal horse serum* intravenously in a dose of 10 ml. per kilogram as recommended by Rich and Gregory.[18] The horse serum was given as a single intravenous injection. Animals subjected to this procedure develop circulating antibodies at a time when residual antigen can be demonstrated in the blood. Immediately after injection of the horse serum, 30 animals were given 2.0 ml. of a suspension containing 50 μg per milliliter of Piromen† intravenously. Piromen is a concentrate of pyrogenic material from a *Pseudomonas* species, the preparation of which has been described.[6] The injection of 100 μg of this material in a normal rabbit is followed by a temperature rise of 2.5 to 3.5° F., this fever lasting for six to eight hours

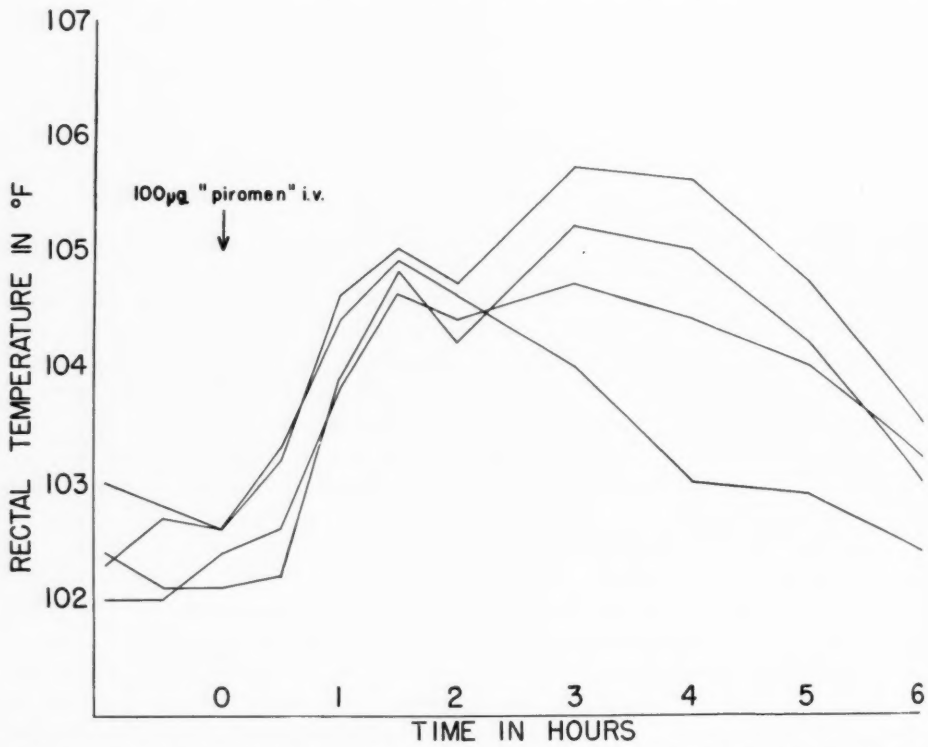


Fig. 1.—Typical temperature responses of normal rabbits to injection of 100 μg of concentrate from *Pseudomonas*.

(Fig. 1). The remaining 30 rabbits received 2.0 ml. of 0.9 per cent NaCl solution intravenously. The saline solutions used for control injections were tested at regular intervals in rabbits and were always pyrogen-free. Daily injections of pyrogen or saline were continued for fourteen days.

Blood was obtained for precipitin tests at three-day intervals after horse serum injection and before animals were sacrificed on the fifteenth day. Serial dilutions of horse serum were layered over undiluted rabbit serum in capillary tubes which were placed upright in a thin layer of modeling clay. After standing at room temperature for eighteen hours, the tests were read with a hand lens using indirect light against a dark background. The last tube

*Kindly supplied by Sharpe and Dohme, Inc., Broad and Wallace Streets, Philadelphia, Pa.
†Kindly supplied by Baxter Laboratories, Inc., Morton Grove, Ill.

in which a white ring of precipitate was visible at the interface was taken as the end point. Serums collected on the fifteenth day were tested for the presence of residual antigen by using anti-horse rabbit serum which yielded a heavy ring with horse serum diluted 1:32,000.

On the fourteenth day after horse serum injection, all animals were given 0.1 ml. of undiluted horse serum intradermally in the flank to test for hypersensitivity. Skin reactions were examined and measured on the fifteenth day immediately before sacrificing the animals.

On the fifteenth day all animals were sacrificed by intravenous injection of Nembutal. At autopsy the thymus and both adrenals were dissected free and weighed. Blocks of heart, lung, liver, spleen, pancreas, both kidneys, adrenal, testis, skeletal muscle, and skin test site were taken for histologic study. Zenker-formol fixation was used, and sections were stained with hematoxylin and eosin.

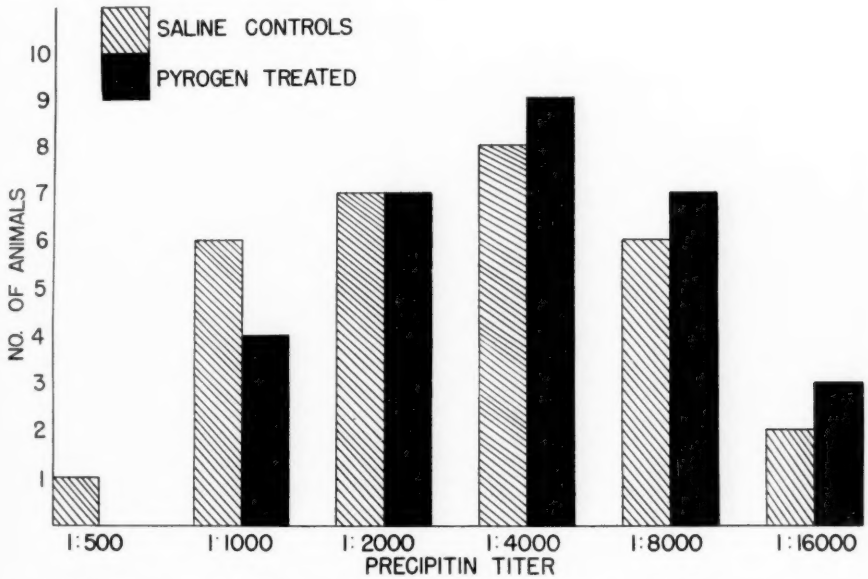


Fig. 2.—Distribution of precipitin titers in rabbits fifteen days after injection of 10 ml. per kilogram of horse serum.

## RESULTS

The incidence of *cardiovascular and renal lesions* in the two groups was entirely comparable. Frank *periarteritis nodosa* occurred in 15 pyrogen-treated animals (50 per cent) and 12 controls (40 per cent). *Endocarditis* was present in 10 of the treated group (33 per cent) and 12 of the saline controls (40 per cent). The incidence of *acute glomerulonephritis* was 43 per cent in animals given pyrogen and 40 per cent in the untreated group. These lesions differed in no way from those which have been described previously as resulting from anaphylactic sensitization of this type.[19-23]

Likewise, *serum antibody* titrations and *skin tests* revealed no essential differences in the two groups. Fig. 2 depicts only the results of precipitin tests performed on serums obtained on the fifteenth day; while there is a slight tendency for the levels in the pyrogen-treated group to be higher than the controls, the difference is not impressive. There was no difference in time of appearance of precipitins during the sensitization period as determined by tests on

serums obtained at three-day intervals, and the results of these earlier tests are not listed. The presence of residual horse serum was demonstrated in all serums on the fifteenth day. The size and appearance of the Arthus reactions at the skin test sites in the two groups were entirely comparable and could not be interpreted as indicating any significant effect of pyrogen treatment upon this reaction (Table I). Histologically, the skin lesions from the two groups were indistinguishable.

TABLE I. SKIN REACTIONS TWENTY-FOUR HOURS AFTER INTRACUTANEOUS INJECTION OF 0.1 ML. OF HORSE SERUM IN SENSITIZED RABBITS

| PIROMEN-TREATED | | | SALINE CONTROLS | | |
|---|---|---|---|---|---|
| RABBIT NO. | EDEMA | HEMORRHAGE | RABBIT NO. | EDEMA | HEMORRHAGE |
| 1 | 2.5 × 3.0 cm. | 1.0 × 1.0 | 31 | 1.0 × 1.0 cm. | 0 |
| 2 | 3.0 × 3.5 | 0 | 32 | 3.5 × 5.0 | 0 |
| 3 | 3.0 × 3.0 | 0 | 33 | 3.0 × 3.5 | 1.0 × 1.0 |
| 4 | 3.0 × 3.0 | 0 | 34 | 2.5 × 3.0 | 0 |
| 5 | 4.0 × 4.0 | 0 | 35 | 2.5 × 3.0 | petechiae |
| 6 | 3.5 × 5.0 | 0 | 36 | 3.0 × 3.5 | petechiae |
| 7 | 3.0 × 3.0 | 0 | 37 | 4.0 × 4.0 | 0 |
| 8 | 2.5 × 3.0 | 0 | 38 | 4.5 × 5.0 | 0 |
| 9 | 3.0 × 4.0 | 0 | 39 | 3.0 × 3.5 | 0.5 × 0.5 |
| 10 | 3.0 × 4.0 | 0 | 40 | 2.5 × 3.0 | 0 |
| 11 | 2.0 × 2.5 | petechiae | 41 | 3.0 × 3.0 | 0 |
| 12 | 3.0 × 3.0 | 0 | 42 | 3.0 × 4.0 | 0 |
| 13 | 2.5 × 3.0 | 0 | 43 | 3.5 × 5.0 | 0 |
| 14 | 5.0 × 5.0 | 1.0 × 1.0 | 44 | 4.0 × 5.0 | petechiae |
| 15 | 2.0 × 2.5 | 0 | 45 | 3.0 × 3.5 | 1.0 × 1.0 |
| 16 | 2.0 × 2.0 | 0 | 46 | 4.0 × 4.5 | 0 |
| 17 | 4.0 × 4.0 | petechiae | 47 | 4.0 × 5.0 | 0 |
| 18 | 2.0 × 2.0 | 0 | 48 | 4.0 × 5.0 | 0.5 × 0.5 |
| 19 | 4.0 × 4.0 | 0 | 49 | 1.0 × 1.0 | 0 |
| 20 | 2.0 × 2.0 | 0 | 50 | 3.0 × 3.5 | 0 |
| 21 | 2.5 × 3.0 | 0 | 51 | 2.0 × 2.5 | 0 |
| 22 | 4.0 × 4.0 | 0 | 52 | 3.0 × 4.0 | 1.5 × 1.5 |
| 23 | 2.5 × 3.0 | 0 | 53 | 4.0 × 5.0 | 0 |
| 24 | 4.0 × 5.0 | petechiae | 54 | 2.0 × 3.0 | 0 |
| 25 | 4.0 × 4.0 | 0 | 55 | 2.0 × 2.5 | 0 |
| 26 | 3.0 × 3.5 | 0 | 56 | 3.0 × 3.5 | 0 |
| 27 | 3.0 × 4.5 | 1.0 × 1.0 | 57 | 2.0 × 3.0 | 0 |
| 28 | 2.0 × 3.5 | 0 | 58 | 2.5 × 4.0 | 0 |
| 29 | 3.0 × 3.5 | 0 | 59 | 4.0 × 4.0 | 0 |
| 30 | 4.0 × 4.5 | 0.5 × 0.5 | 60 | 2.0 × 3.0 | 0 |
| Average | 3.0 × 3.4 | | Average | 2.9 × 3.6 | |

Table II compares the weights of *adrenals* and *thymus glands* in the two groups of animals. Animals given Piromen showed no evidence of the adrenal hypertrophy or thymic atrophy characteristic of rabbits given ACTH.[24, 25, 28] The *spleens* of animals in each group showed spread of lymphoid tissue into the pulp and peri-Malpighian body lymphoid hyperplasia of the type described by Rich, Lewis, and Wintrobe[29] in animals given foreign protein parenterally. No evidence of modification of the process was noted in pyrogen-treated animals. Inhibition of this "acute splenic tumor" has been observed in sensitized rabbits given ACTH[24, 25] or cortisone.[26] There was no detectable difference in glycogen or fat distribution in the *livers* of the two groups of animals. ACTH often produces an increase in these substances in the hepatic cells and cortisone produces a striking increase in hepatic glycogen.[24-26]

TABLE II. BODY WEIGHT AND WEIGHT OF ADRENALS AND THYMUS OF PIROMEN-TREATED AND CONTROL ANIMALS AT AUTOPSY

| PIROMEN-TREATED | | | | SALINE CONTROLS | | | |
|---|---|---|---|---|---|---|---|
| RABBIT | BODY WT. (GM.) | ADRENALS (GM.) | THYMUS (GM.) | RABBIT | BODY WT. (GM.) | ADRENALS (GM.) | THYMUS (GM.) |
| 1 | 2,220 | 0.32 | 3.12 | 31 | 2,150 | 0.20 | 2.62 |
| 2 | 2,140 | 0.27 | 3.72 | 32 | 1,920 | 0.22 | 3.81 |
| 3 | 2,175 | 0.25 | 4.21 | 33 | 2,100 | 0.24 | 2.84 |
| 4 | 2,000 | 0.28 | 2.40 | 34 | 2,250 | 0.27 | 2.09 |
| 5 | 1,920 | 0.19 | 1.96 | 35 | 1,890 | 0.21 | 1.35 |
| 6 | 2,120 | 0.19 | 1.29 | 36 | 1,930 | 0.15 | 1.57 |
| 7 | 2,520 | 0.25 | 4.99 | 37 | 1,975 | 0.19 | 0.90 |
| 8 | 2,550 | 0.21 | 3.35 | 38 | 2,150 | 0.24 | 1.54 |
| 9 | 2,290 | 0.18 | 3.01 | 39 | 2,050 | 0.24 | 3.84 |
| 10 | 1,860 | 0.14 | 1.08 | 40 | 2,300 | 0.19 | 2.80 |
| 11 | 1,965 | 0.16 | 2.60 | 41 | 2,495 | 0.20 | 2.60 |
| 12 | 2,250 | 0.21 | 3.20 | 42 | 2,530 | 0.20 | 2.90 |
| 13 | 2,030 | 0.27 | 3.10 | 43 | 2,120 | 0.21 | 2.62 |
| 14 | 2,045 | 0.26 | 2.40 | 44 | 2,050 | 0.19 | 3.10 |
| 15 | 2,380 | 0.27 | 2.10 | 45 | 2,210 | 0.25 | 3.96 |
| 16 | 2,230 | 0.23 | 3.26 | 46 | 2,090 | 0.19 | 1.81 |
| 17 | 2,055 | 0.16 | 2.00 | 47 | 2,505 | 0.24 | 3.76 |
| 18 | 2,150 | 0.36 | 3.79 | 48 | 1,890 | 0.21 | 2.80 |
| 19 | 2,185 | 0.20 | 3.40 | 49 | 2,110 | 0.30 | 4.10 |
| 20 | 2,275 | 0.24 | 2.77 | 50 | 2,610 | 0.23 | 3.65 |
| 21 | 2,350 | 0.16 | 4.71 | 51 | 2,020 | 0.24 | 2.52 |
| 22 | 2,080 | 0.33 | 1.70 | 52 | 2,700 | 0.32 | 6.12 |
| 23 | 2,220 | 0.34 | 2.74 | 53 | 2,455 | 0.17 | 3.12 |
| 24 | 1,820 | 0.21 | 1.78 | 54 | 2,105 | 0.22 | 3.45 |
| 25 | 1,685 | 0.17 | 1.94 | 55 | 1,815 | 0.19 | 1.96 |
| 26 | 1,750 | 0.20 | 2.46 | 56 | 1,790 | 0.26 | 2.30 |
| 27 | 1,890 | 0.19 | 2.30 | 57 | 2,245 | 0.24 | 3.46 |
| 28 | 2,015 | 0.22 | 3.74 | 58 | 2,310 | 0.19 | 1.98 |
| 29 | 2,320 | 0.26 | 3.42 | 59 | 2 145 | 0.22 | 2.04 |
| 30 | 1,950 | 0.23 | 2.88 | 60 | 2,080 | 0.24 | 2.71 |
| Range | 1,685-2,380 | 0.14-0.36 | 1.08-4.99 | | 1,790-2,610 | 0.15-0.32 | 0.9-4.1 |
| Mean | 2,114 | 0.231 | 2.84 | | 2,166 | 0.222 | 2.81 |

Last, the *adrenals* of pyrogen-treated animals showed no depletion of cortical lipoid; indeed the histologic appearance of the glands from both groups of rabbits was the same. Loss of cortical lipoid after stimulation by ACTH was pointed out by Sayers and Sayers[30] as a characteristic change and has been observed in sensitized rabbits given ACTH.[24, 25]

## DISCUSSION

The incidence of cardiovascular lesions and glomerulonephritis in the animals given Piromen justifies the conclusion that this material, in the dosage employed, exerted no inhibitory effect upon serum disease in rabbits. Because ACTH has been shown to be very effective in decreasing the incidence of these lesions in anaphylactically sensitized animals, this investigation furnishes no support for the idea that bacterial pyrogens exert an ACTH-like action. The absence of other morphologic changes known to result from administration of ACTH to sensitized rabbits is further evidence against significant adrenal stimulation by the pyrogen.

There was no clear-cut evidence of stimulation of antibody formation such as Stuart noted.[8] Likewise, the absence of significant differences in skin re-

actions to horse serum in the two groups is in contrast to the enhancement of the Arthus phenomenon reported by Stuart. However, Stuart's animals were not sensitized by a single injection of a large amount of antigen which would result in the presence of residual circulating antigen at the time of testing. Furthermore, Stuart's animals received a much smaller dose of pyrogen than did those in the present study. Within limits, the severity of the Arthus reaction is proportional to antibody titer,[38] and, as previously mentioned, at the time the animals were skin-tested in this study, the titers were essentially the same.

## SUMMARY

Of 60 rabbits sensitized with horse serum, 30 were given 14 daily injections of a pyrogenic *Pseudomonas* concentrate, the remainder serving as controls. At autopsy, no difference in the incidence of cardiovascular and renal lesions was found in the two groups. There were no impressive differences in precipitin titers or skin reactions to horse serum in pyrogen-treated and control animals.

It is concluded that, in the dosage used in this study, the pyrogen exerted no significant adrenocortical stimulatory effect. This is based on the known efficacy of ACTH in suppressing the development of cardiovascular and renal lesions in anaphylactically sensitized animals, as well as the absence in pyrogen-treated animals of characteristic morphologic changes which result from ACTH administration.

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# A METHOD OF QUANTITATING 1-HYDRAZINOPHTHALAZINE IN BODY FLUIDS

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THE elevated blood pressures of hypertensive patients are now being effectively controlled with a combination of 1-hydrazinophthalazine (C-5968) and hexamethonium halide.[1, 2] With the advent of this therapy, it became desirable to quantitate both compounds in body fluids. Since the hexamethonium ion had had considerable previous therapeutic use,[3-5] an adequate method was available for measuring it.[6] Despite prior clinical trial,[7] however, no way of estimating hydrazinophthalazine had been devised. This drug consists of the heterocyclic phthalazine ring with an attached hydrazine group. The hydrazine moiety is the most reactive part of the molecule. It has considerable affinity for sulfhydryl and carbonyl radicals; it combines with heavy metals; with heat it is bound by glucose but not by sucrose; and it forms a highly colored complex on warming with ninhydrin in an acid medium.

## METHOD

Advantage is taken of the last reaction in quantitating the compound. The method depends upon the measurement of the fraction of the hydrazinophthalazine which is extracted from water into chloroform as a ninhydrin complex. The procedure for urine is as follows: A 100 ml. aliquot is brought to pH 3.0 ± 0.2 and an ionic strength of about 0.5 exclusive of urinary electrolytes with glacial acetic acid and, when needed, sodium acetate. 0.50 ml. of 2.4 per cent ninhydrin (triketohydrindine hydrate) is added; we have found isopropanol more satisfactory as a ninhydrin solvent than water. The mixture is heated to 70° C. for seven minutes, and then immediately cooled in an ice bath after which it is extracted by means of vigorous shaking for at least thirty seconds with 10 ml. of chloroform. When the two liquids have partially separated, the chloroform layer which contains the yellow ninhydrin-hydrazinophthalazine complex is cleared by centrifugation. The color is stable for at least one hundred hours at 5° C. The amount of hydrazinophthalazine in the urine is linearly proportional to the 460 mμ optical density of the chloroform solution as measured by the Beckman photoelectric spectrophotometer. If the solution is too opaque to read, it can be diluted with chloroform as much as necessary. Under the conditions set forth above the following equation is a good approximation:

$$\text{micromolarity of urinary C-5968} = 7.7\ (460\ m\mu \text{ optical density} - 0.10)$$

It is important that extraction be completed relatively rapidly. When agitation is repeated at intervals, abnormally high values are obtained. Only the ninhydrin-hydrazinophthalazine complex is extracted into chloroform. At equilibrium the greater part of each reactant is uncombined, and both of the free compounds remain almost entirely in the aqueous layer. After removal of the colored complex, a mass action effect causes more to be formed

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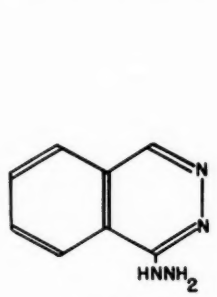
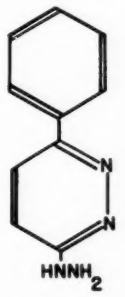
which can also be extracted. There is no appreciable tendency, however, for the back reaction to occur in chloroform at room temperature; consequently the optical density of the complex remains constant.

Some yellow urinary pigment passes from the water to the chloroform layer at pH 3.[8] If necessary, urine can be cleared of this interfering material by serial batch extractions of the pigment with chloroform before the ninhydrin is added. Since the free hydrazinophthalazine is extremely hydrophilic, it is affected only very slightly by this procedure. Ordinarily such initial extraction is superfluous unless the greatest possible accuracy is desired. Extractible pigment color at 460 mμ is seldom one-twentieth as great as the color of the ninhydrin complex even when a patient is on relatively small doses.

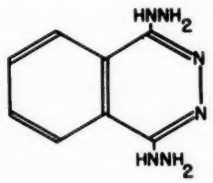
At room temperature the drug is stable in distilled water for weeks; whereas in urine it quickly disappears. Even at 5° C., forty-eight hours is sufficient to clear the urine almost completely; however, freezing at subzero temperatures does halt decomposition. In practical terms this means that urine should be assayed or frozen immediately after voiding; even then losses may occur in the urinary tract.



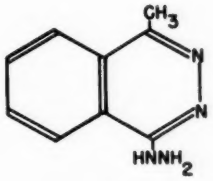

C-5968




C-6084




C-7441




C-6130

Fig. 1.—Chemical formulas of 1-hydrazinophthalazine (C-5968), 1,4-dihydrazinophthalazine (C-7441), 1-hydrazino-4-methylphthalazine (C-6130), and 3-hydrazino-6-phenyl-1,2-diazine (C-6084).

The method is applicable to blood. The difficulties involved are due to small initial volume and low concentration. To avoid dilution the cells are removed by centrifugation and the proteins precipitated by solid trichloracetic acid. Thereafter the procedures used with urine can be directly applied. If necessary, essentially all the color developed can be concentrated in 1/25 or even 1/100 volume of chloroform.

## DISCUSSION

The absorption spectrum of the ninhydrin-hydrazinophthalazine complex in chloroform has a single major peak at 460 mμ. In water the peak is at 430 mμ. The maximum molecular extinction coefficient of the hydrochloride is $1.31 \times 10^4$ if it is colored by the procedure outlined above.

Of some clinical interest are three chemically related experimental drugs: 1-hydrazino-4-methylphthalazine (C-6130); 1,4-dihydrazinophthalazine (C-7441); and 3-hydrazino-6-phenyl-1,2-diazine (C-6084) (Fig. 1). All have single peaked, and therefore somewhat similar, absorption spectra. The first (C-6130) has a maximum molecular extinction coefficient of $1.37 \times 10^4$ at 460 m$\mu$; for the second (C-7441) the maximum coefficient is $1.50 \times 10^4$ at 480 m$\mu$; and for the third (C-6084) it is $6.0 \times 10^3$ at 375 m$\mu$ (Fig. 2). Phenylhydrazine gives a distinctive two-peaked curve with maxima at 395 and 430 m$\mu$ where the molecular extinction coefficients are $2.3 \times 10^3$ and $1.8 \times 10^3$, respectively. Hydrazine, itself, yields an absorption spectrum with only one peak. It is at 375 m$\mu$, and the corresponding extinction coefficient is only $3.8 \times 10$.

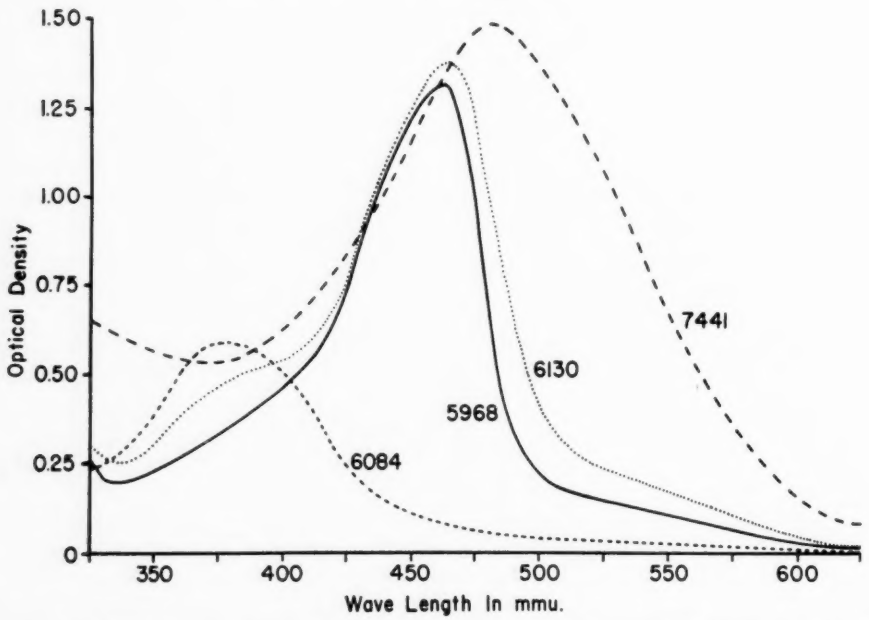



Fig. 2.—Absorption spectra of the ninhydrin complexes formed as described with 50 $\mu$M of 1-hydrazinophthalazine hydrochloride (C-5968), 50 $\mu$M of 1-hydrazino-4-methylphthalazine hydrochloride (C-6130), 50 $\mu$M of 1,4-dihydrazinophthalazine dihydrochloride (C-7441), and 50 $\mu$M of 3-hydrazino-6-phenyl-1,2-diazine hydrochloride (C-6084), all of which were kindly supplied by Ciba Pharmaceutical Products.

The 460 m$\mu$ optical density is a function of pH over the range of 1 to 10; neglecting specific anion effects, pH 3 gives maximal color. Increasing acidity causes a more rapid diminution in opacity than increasing alkalinity.

Again neglecting specific ion effects, the 460 m$\mu$ opacity depends on ionic strength. For an acetic acid-acetate buffer system an ionic strength of one-half gave maximal color, but electrolyte concentration is not critical until it reaches very high values.

Finally the 460 m$\mu$ optical density varies with the time and temperature of heating. No maxima occur but the optical density approaches a limit as the warming period becomes longer and hotter. The standard procedure uses a

convenient, albeit arbitrary, time and temperature where these variables are not critical. The opacity corresponding to heating at 70° C. for seven minutes, although reproducible, is less than half of the limiting value obtained by boiling for forty minutes.

The optical density of colored complex for given ninhydrin and hydrazinophthalazine concentrations can be predicted accurately. The reaction between ninhydrin and hydrazinophthalazine is apparently a simple reversible dimolecular one. The best value of the equilibrium constant $\frac{[N] \times [5968]}{[N5968]}$

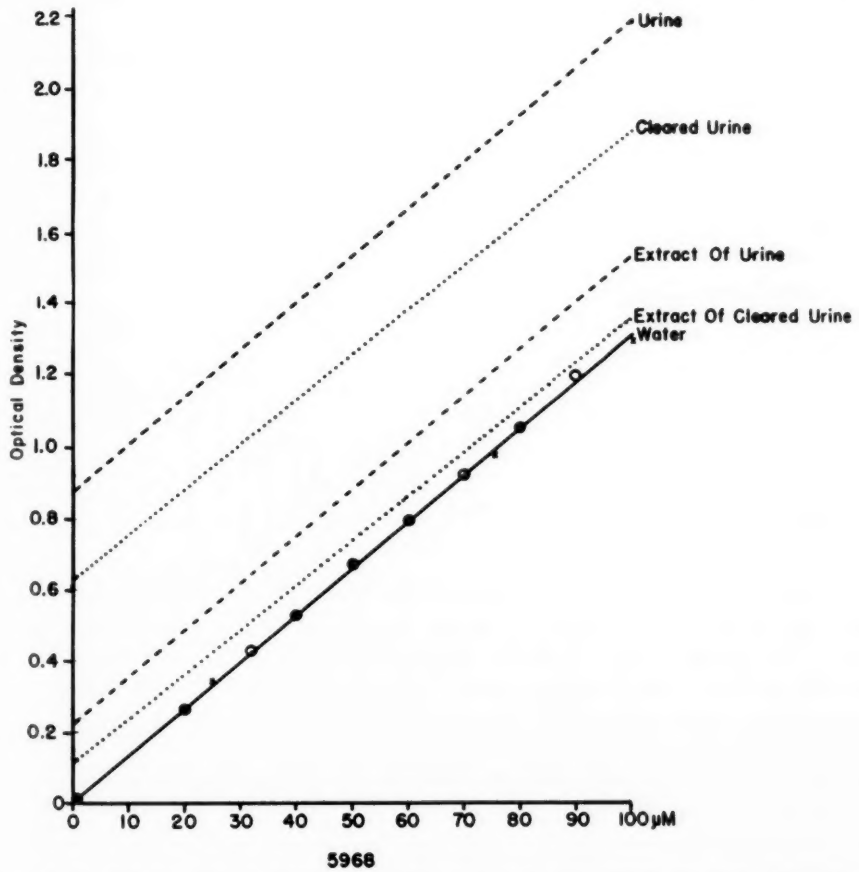



Fig. 3.—460 mμ optical density as a function of 1-hydrazinophthalazine concentration under varying conditions. The solid line labeled water was obtained when the complex was formed in distilled water. It represents three superimposed curves. The first was obtained by reading the water directly (×); the next by extracting it with an equal volume of chloroform and reading the chloroform (o); the last by extracting it with one-tenth volume of chloroform, then diluting to 10 with chloroform, and reading the chloroform (●). For the other four curves the complex was formed in urine; the two dashed lines represent urine that was not pretreated; the two dotted lines represent urine that was extracted at pH 3 with one-half volume of chloroform after the hydrazinophthalazine had been added but before the ninhydrin was put in. As indicated the urines were either read directly or else extracted with equal volumes of chloroform. For the sake of clarity the graphs representing extraction in one-tenth volume of chloroform were omitted; however, if the chloroform extracts are diluted with nine volumes more of chloroform the curves are parallel to their respective parent curves. Their intercepts on the vertical axis were 0.103 for the uncleared and 0.017 for the cleared urine used in this particular experiment. The slightly decreased slope of the pre-extracted urine and its extracts is due to the removal of free hydrazinophthalazine by chloroform.

is 1375 (Table I), with a standard deviation of 104. The theoretical deviation of the concentration curve (Fig. 3) from linear due to increasing dissociation over the range 0 to 100 $\mu$M C-5968 is about 3 per cent; this is not obvious experimentally. Since the reaction is not nearly complete, the previously mentioned danger of its proceeding further is very real if the equilibrium is upset by extraction of the product. The reappearance of a yellow color in the aqueous layer is easy to demonstrate after selective removal of the complex by shaking with chloroform.

TABLE I. DETERMINATION OF EQUILIBRIUM CONSTANT OF NINHYDRIN-HYDRAZINOPHTHALAZINE COMPLEX

*460 m$\mu$ Optical Density Reading × Dilution Factor*

| Ninhydrin \ C-5968 | 25 | 50 | 100 | 200 | 400 $\mu$M |
|---|---|---|---|---|---|
| 2400 $\mu$M | | | 64.95 | | |
| 1200 | | 22.40 | 45.20 | 84.90 | |
| 600 | 7.481 | 14.10 | 30.46 | 56.00 | 164.0 |
| 300 | | 8.040 | 18.90 | 35.13 | |
| 150 | | | 9.581 | | |

$$K = [N] \times [H]/[NH]$$

| Ninhydrin \ C-5968 | 25 | 50 | 100 | 200 | 400 $\mu$M | Mean |
|---|---|---|---|---|---|---|
| 2400 $\mu$M | | | 1260 | | | 1260 |
| 1200 | | 1450 | 1401 | 1513 | | 1455 |
| 600 | 1387 | 1499 | 1301 | 1397 | (628) | 1403* |
| 300 | | 1524 | 1206 | 1243 | | 1324 |
| 150 | | | 1325 | | | 1293 |
| Mean | 1387 | 1491 | 1298 | 1384 | | 1375* |

*Value in parentheses omitted.

Extraction of the yellow ninhydrin-hydrazinophthalazine complex from water at pH 3 into chloroform can be demonstrated visually to be very complete; the distribution coefficient approximates 500. This very high value permits extreme concentration of the complex in the chloroform coupled with essentially complete recovery. At the same time there is almost no removal of the uncombined compounds from the aqueous phase due to their hydrophilia and the greater volume of this phase. At pH 3 free ninhydrin has a water-chloroform distribution coefficient of about ten while free hydrazinophthalazine has an even higher one.

The almost parallel straight lines shown in Fig. 3 are plots of 460 m$\mu$ optical density versus 1-hydrazinophthalazine concentration under varying conditions. The curve of concentration in water versus optical density passes through the origin of the graph. Extracting with chloroform does not change the curve even if the volumes of the two phases are different, provided the chloroform readings are corrected. When the ninhydrin reaction occurs in urine and color is measured directly, a non-zero blank appears but the slope is unaltered. Chloroform extraction either of the original urine before ninhydrin is added or of the colored urine reduces the blank. The slight diminution in

slope with pre-extraction is presumably due to the minimal removal of hydrazinophthalazine along with urochrome and related substances. In any case the final color depends only on urinary drug level and blank reading. With care the method as described is capable of better than 3 per cent accuracy for concentrations greater than 10 micromolar, and this can be considerably improved by such devices as pre-extraction and bracketing of unknown readings by known standards. The precision is less for lower concentrations, but under suitable conditions one micromolar or even more dilute solutions can be estimated.

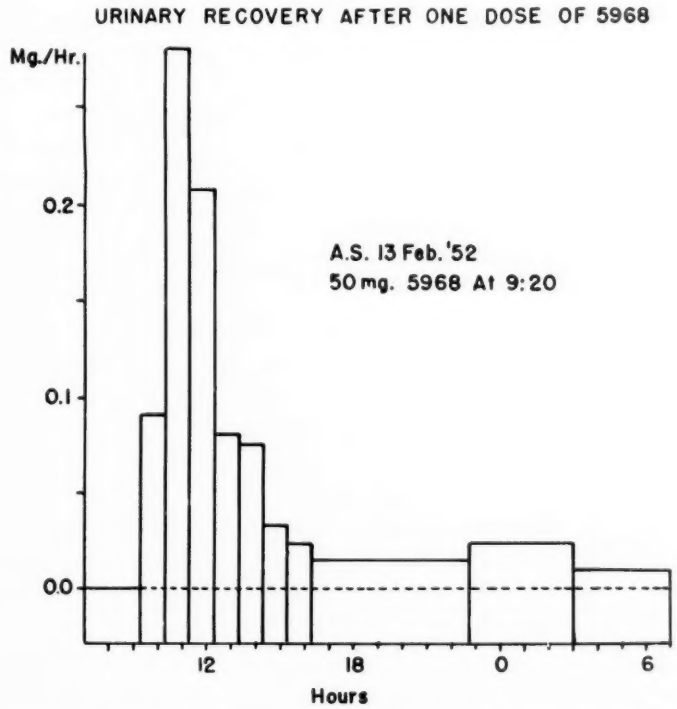



Fig. 4.—Twenty-four hour urinary excretion in mg. · $hr^{-1}$ of 1-hydrazinophthalazine (C-5968) after a single oral dose of 50 mg. of the drug. The patient was a 51-year-old woman with moderately severe neurogenic hyptertension for which she had not been previously receiving this drug. The area below the dotted line represents the urinary pigment found in the urine voided before taking the drug.

As was mentioned previously, the hydrazine moiety of the molecule reacts with ketone groups, sulfhydryl radicals, many heavy metal ions, glucose, and ninhydrin. In vitro experiments reveal that water-soluble mercaptans are bound by 1-hydrazinophthalazine but not by phthalazone where the hydrazine is replaced by oxygen. For molecules with three or more carbon atoms the ability to combine is therefore dependent on the presence of a hydrophilic group; hence n-propyl mercaptan remains uncombined whereas ethyl mercaptan and $\beta$-mercapto propionic acid are bound. Hydroxyl groups cannot substitute for sulfhydryl groups, as evinced by the fact that serine is inert and cysteine active. Some binding of hydrazinophthalazines, probably involving glutathione and

perhaps other related compounds, occurs in vivo since it is possible to break the combination and then recover additional previously bound hydrazinophthalazine from urine. The linkage between sulfhydryl groups and certain metallic salts, such as antimony trichloride for instance, is tighter than the mercaptohydrazine linkage and under suitable conditions replaces it. Since the drug combines with many metals, however, the latter must be used cautiously. Preliminary results show that patients on C-5968 uniformly have both free and bound compound in their urines with the amount of the latter that can be measured ranging from a very small fraction to two or three times that of the free drug.

Carbonyl as well as sulfhydryl groups react with hydrazinophthalazine but not with phthalazone. Solubility in water is again a requisite to activity; hence acetaldehyde combines; whereas phenylacetaldehyde does not. Like $\beta$-mercaptopropionic acid, pyruvic acid is strongly bound; surprisingly enough the effect of the two groups seems additive in $\beta$-mercaptopyruvic acid. Acetone on the other hand seems to be entirely without activity. Although it has not been possible to break the linkage between carbonyl and hydrazine radicals, there is evidence that the linkage occurs in vivo. Preliminary data, however, indicate that blood pyruvic acid levels are not depressed in patients on C-5968 therapy.

The common alkali metals and alkaline earths of body fluids: sodium, potassium, magnesium, and calcium, as well as the heavier caesium and barium, do not combine with hydrazinophthalazine. On the other hand, ferric, cupric, manganous, and cobaltous ions comprise a group of physiologic metals which do; the first pair is much more active than the second. Whereas the ferric ion is very reactive, the ferrous ion has no activity.

The reaction of hydrazinophthalazine with glucose is apparently analogous to osazone formation with phenylhydrazine. It presumably is of no physiologic importance since it occurs only after long boiling with concentrated sugar solutions.

Clinical studies will be more fully discussed in a later communication. In general, the procedure reveals a noticeable urine concentration of the hydrazinophthalazine within thirty minutes of a single oral dose; a maximum is reached during the second to fourth hour, thereafter the drug is slowly excreted for more than twenty-four hours. Fig. 4 shows the typical time course of urinary excretion of C-5968 after 50 mg. by mouth. The low value in the evening urine is probably due to the fact that assay was postponed until twelve hours after voiding, allowing appreciable drug decomposition. To date there has been a surprising uniformity in the percentage of drug recovered; whether it is given orally or parenterally the recovery of free drug approximates $2 \pm \frac{1}{2}$ per cent.

## SUMMARY

A colorimetric method is described for the determination of 1-hydrazinophthalazine or related substances as ninhydrin complexes in urine or plasma. Its difficulties are discussed and its dependence on reaction variables outlined. Five compounds or groups with which the drug reacts are mentioned:

(1) sulfhydryl radicals, (2) carbonyl radicals, (3) glucose, (4) heavy metal ions, (5) ninhydrin.

I wish to acknowledge the able technical assistance of Mrs. Robert Goldstein and Mrs. Michael Mahoney. I am indebted to Elizabeth F. Perry for the illustrations and to Dr. Henry A. Schroeder for his aid in the preparation of the manuscript. The four experimental drugs were kindly furnished us by Ciba Pharmaceutical Products.

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# THE INFLUENCE OF ANTIHISTAMINIC AGENTS ON ERYTHROCYTE AGGLUTINATION AND RELATED PHENOMENA

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THE addition of certain antihistaminic agents to whole blood prior to its infusion has significantly lowered the incidence of untoward transfusion reactions.[1, 2] This is of particular importance for those patients who require multiple and repeated transfusions.[3] During a study of this phenomenon, it was observed that the addition of Benadryl to anti-A and anti-B sera significantly retarded the specific agglutination in vitro of Group A and Group B corpuscles, respectively. This report deals with further analyses and observations on the inhibiting effect of Benadryl and other antihistaminic agents on isoagglutination of human blood cells in vitro.

## METHODS

1. Fresh erythrocytes of blood groups A and B were washed three times in 0.9 per cent NaCl solution and resuspended to a concentration of 1.5 to 2.0 per cent.

2 (a). Equal parts (0.1 ml each) of sera (anti-A or anti-B) and the selected antihistaminic agent (as commercially available) were mixed together in a small test tube and vigorously shaken for one minute. In one depression of a microslide was placed 1 drop of the serum-antihistaminic mixture, and in the other 1 drop of a serum-saline mixture which served as a control. One drop of the appropriate cell suspension (Group A or Group B) was added to each, and the slide was rotated to insure adequate mixing of the components.

2 (b). To 0.5 ml. of Group A or Group B cell suspension in a small test tube was added 0.1 ml. of an antihistaminic agent (as commercially available) and the mixture was vigorously shaken for one minute. In one depression of a microslide was placed 1 drop of the cell suspension-antihistaminic agent mixture and in the companion depression 1 drop of the cell suspension in saline solution which served as a control. To each was added one drop of the appropriate antiserum (anti-A or anti-B), and the slide was rotated to ensure mixing of the cell suspension and serum.

3. The time of agglutination of series of mixtures was recorded by stopwatch from the instant of contact between the serum and red cell suspension. The mixtures were observed under low power at intervals not greater than thirty seconds in order to detect the initiation and degree of clumping of the cells. The slide, while on the stage of the microscope, was gently agitated at one-minute intervals in order to promote clumping.

4. Agglutination reactions were recorded as ±, 1+, 2+, 3+, or 4+ (4+ indicated the presence of large clumps, although some free cells might still be present).

5. Additional controls consisted of appropriate samples of the blood in 0.9 per cent saline diluent and in Phemerol preservative, diluted 1 to 10,000.

6. In order to determine the effect of prolonged contact of an antihistaminic agent with cell suspension or antiserum, the cell suspension and antihistaminic agents, as well as the mixtures of anti-A or anti-B sera and antihistaminic agents, were incubated for varying periods of time before testing for any evidence of inhibition.

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TABLE I. SUMMARY OF THE EFFECTS OF EIGHT ANTIHISTAMINIC PREPARATIONS ON ISOAGGLUTINATION, LISTED IN ORDER OF THEIR INHIBITING ACTIVITY

| ANTIHISTAMINE | CONCENTRATION | PREPARATION | OTHER INGREDIENTS CONTAINED IN PREPARATION | BRAND | EFFECT ON ISOAGGLUTINATION: TREATED CELLS | EFFECT ON ISOAGGLUTINATION: TREATED SERA |
|---|---|---|---|---|---|---|
| Neo-antergan (Pyranisamine maleate) | 10 mg./c.c. | Crystalline | ? | Merck | Moderate initial inhibition | Marked prolonged inhibition |
| Benadryl hydrochloride (Dipenhydramine hydrochloride) | 10 mg./c.c. | liquid | Phemerol 1-10,000 | Parke-Davis | Moderate initial inhibition | Marked initial inhibition |
| Pyribenzamine hydrochloride (Tripelenamine hydrochloride) | 25 mg./c.c. | liquid | ? | Ciba | Very slight inhibition | Moderate inhibition |
| Histadyl (Thenylpyramine) | 20 mg./c.c. | liquid | Chlorobutanol 0.5% | Lilly | Very slight inhibition | Moderate inhibition |
| Antistine hydrochloride (Phenazoline hydrochloride) | 0.5% | liquid | ? | Ciba | No inhibition | Very slight inhibition |
| Neosynephrine (Thenfadil hydrochloride) | 0.25% Neosynephrine hydrochloride 0.1% Thenfadil hydrochloride | liquid | Methylparoben 0.025% Propylparoben 0.014% Sodium bisulfite 0.2% | Winthrop-Stearns | No inhibition | No inhibition |
| Clopane hydrochloride (Cyclopentamine hydrochloride) | 0.5% | liquid | Phenylmercuric nitrate 1-50,000 | Lilly | No inhibition | No inhibition |
| Dramamine (Dimenhydrinate) | 25 mg./8 c.c. | liquid | Alcohol 5% | Searle | No inhibition | No inhibition |

Note: Degrees of inhibition of isoagglutination:

Marked inhibition = 600 to 1200 seconds.
Moderate inhibition = 300 to 600 seconds.
Slight inhibition = 180 to 300 seconds.
Very slight inhibition = 60 to 180 seconds.

RESULTS

A. *Comparative Tests With Eight Antihistaminic Preparations.*—Eight antihistaminic agents were tested to determine their relative abilities to inhibit isoagglutination* (Table I). Since some of the antihistaminic agents contained undisclosed ingredients which were not available for study and which may possibly have interfered with their inhibitory action, it was difficult to make definitive comparisons of all preparations. In the cases in which some inhibitory activity was observed, the 4+ reaction usually occurred abruptly following varying time intervals during which small aggregates of rouleaux were seen which became dispersed when the slide was gently agitated. The results of the tests indicate that Pyranisamine maleate, diphenhydramine HCl and tripelenamine HCl exhibited the greatest inhibition of agglutination.

B. *Tests in Which Anti-A or Anti-B Sera Were Treated With Antihistaminic Agents Prior to Combining With Group Specific Erythrocytes.*—Pyranisamine maleate showed the greatest inhibition of isoagglutination among the

TABLE II. THE REACTION TIME OF AGGLUTINATION OF ANTIHISTAMINIC-TREATED ANTI-A OR ANTI-B SERA WHEN MIXED WITH SUSPENSIONS OF GROUP SPECIFIC ERYTHROCYTES

| HYPERIMMUNE ANTISERA + EQUAL PARTS OF | LENGTH OF INCUBATION OF SERUM AND DILUENT: | ANTI-A SERUM + GROUP A CELLS | | ANTI-B SERUM + GROUP B CELLS | |
|---|---|---|---|---|---|
| | | FIRST APPEARANCE OF AGGLUTINATION (± TO 1+) | APPEARANCE OF LARGE CLUMPS (4+) | FIRST APPEARANCE OF AGGLUTINATION (± TO 1+) | APPEARANCE OF LARGE CLUMPS (4+) |
| Saline | none | 30 sec. | 90 sec. | 30 sec. | 90 sec. |
| | 1 hour* | 30 | 90 | 30 | 60 |
| | 3 days† | 30 | 60 | 30 | 90 |
| Phemerol 1-10,000 | none | 30 | 90 | 30 | 90 |
| | 1 hour | 30 | 90 | 30 | 60 |
| | 3 days | 30 | 60 | 30 | 90 |
| Neo-antergan (Pyranisamine maleate) | none | 450 | 840 | 420 | 870 |
| | 1 hour | 540 | 1140 | 720 | 1140 |
| | 3 days | 240 | 540 | 240 | 450 |
| Benadryl | none | 300 | 900 | 180 | 330 |
| | 1 hour | 330 | 390 | 360 | 510 |
| | 3 days | 180 | 240 | 180 | 240 |
| Pyribenzamine | none | 300 | 510 | 270 | 420 |
| | 1 hour | 210 | 270 | 210 | 270 |
| | 3 days | 210 | 660 | 210 | 240 |
| Histadyl | none | 270 | 360 | 270 | 300 |
| | 1 hour | 270 | >630 | 270 | 360 |
| | 3 days | 570 | 630 | 270 | 360 |
| Neosynephrine thenfadil | none | 30 | 240 | 30 | 150 |
| | 1 hour | 30 | 150 | 30 | 60 |
| | 3 days | 60 | 120 | 60 | 90 |
| Antistine | none | 90 | 120 | 90 | 120 |
| | 1 hour | 60 | 90 | 60 | 90 |
| | 3 days | 60 | 90 | 60 | 90 |
| Clopane hydrochloride | none | 60 | 570 | 60 | 300 |
| | 1 hour | 60 | 210 | 60 | 150 |
| | 3 days | 60 | 720 | 60 | 240 |
| Dramamine | none | 30 | 60 | 30 | 60 |
| | 1 hour | 30 | 60 | 30 | 60 |
| | 3 days | 30 | 60 | 30 | 90 |

*One hour at room temperature.

†Three days at 4° C. Serum mixtures were then brought to room temperature and mixed with appropriate cell suspensions.

*The commercially available concentrations of the antihistaminic agents were used in the tests.

TABLE III. THE REACTION TIME OF ANTIHISTAMINE-TREATED NORMAL GROUP O SERA WHEN MIXED WITH GROUP A AND GROUP B ERYTHROCYTES

| GROUP "O" SERA + EQUAL PARTS OF | LENGTH OF INCUBATION OF "O" SERUM AND DILUENT: | GROUP A CELLS | | | | GROUP B CELLS | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | AGGLUTINATION | | HEMOLYSIS | | AGGLUTINATION | | HEMOLYSIS | |
| | | ± TO 1+ | 4+ | BEGINNING | COMPLETE | ± TO 1+ | 4+ | BEGINNING | COMPLETE |
| 0.9% Saline | no incubation | 60 sec. | 150 sec. | no hemol. | no hemol. | 60 sec. | 150 sec. | no hemol. | no hemol. |
| | 1 hour* | 60 | 90 | no hemol. | no hemol. | 60 | 90 | no hemol. | no hemol. |
| Phemerol 1-10,000 | no incubation | 60 | 120 | no hemol. | no hemol. | 60 | 120 sec. | no hemol. | no hemol. |
| | 1 hour* | 60 | 90 | no hemol. | no hemol. | 60 | 90 | no hemol. | no hemol. |
| Benadryl 1% | no incubation | no aggl. | no aggl. | 90 sec. | 330 sec. | 570 | 930† | no hemol. | no hemol. |
| | 1 hour* | no aggl. | no aggl. | 90 | 480 | no aggl. | no aggl. | 90 sec. | 720 sec. |
| Neo-antergan | no incubation | 180 | 900‡ | 540 | 810 | 900 | 1320 | none | none |
| (Pyranisamine | 1 hour* | none | none | 540 | >1140 | 1020 | 1320 | 300 | 900§ |
| maleate) | no incubation | none | none | 60 | 570 | 600 | 900‡ | 60 | 780 |
| Pyribenzamine | 1 hour* | none | none | 60 | 420 | none | none | 60 | 540 |

*At room temperature.
†Many unagglutinated cells were still present although some large clumps were observed.
‡The clumping observed was slight (1+ or ±) and a 4+ reaction was not observed.
§Complete hemolysis did not occur.

antihistaminic agents tested (Table II). Pyranisamine maleate, however, was the only test substance which was obtained in crystalline form—the others were in solution and contained other ingredients which may have interfered in some way with the reactions. There was no hemolysis observed either in the tests or the controls when hyperimmune anti-A and anti-B sera were used as the source of agglutinins.

When normal Group O sera were used as the source of anti-A and anti-B antibodies, hemolysis of varying intensities occurred (Table III). Slight hemolysis was detected with Benadryl and Pyribenzamine within sixty to ninety seconds; with Pyranisamine maleate hemolysis did not occur in less than 540 seconds. In the instances in which hemolysis occurred without evidence of preliminary agglutination of the cells, it may be assumed that direct lysis took place.

C. *Tests in Which Erythrocyte Suspensions Were Treated With Benadryl Prior to Mixing With the Specific Anti-A or Anti-B Antisera.*—The agglutination time of Benadryl-treated cells was contrasted with (a) untreated cells and (b) cells treated with Phemerol 1 to 10,000. The addition of Benadryl to a saline suspension of erythrocytes caused a delay in the agglutination of the cells with group specific antisera. This effect was most noticeable when the cell-suspension-Benadryl mixture was immediately added to the antiserum without a previous period of incubation (Table IV). The delay in agglutination was approximately 165 seconds for the initial appearance of small clumps (± to 1+ reaction) and approximately 240 to 312 seconds for large (macroscopic, 4+) clumps to form. A concentrated solution of Phemerol (1 to 1,000 dilution) was also used in order to determine whether a high concentration of this substance might inhibit agglutination, but unlike the 1 to 10,000 dilution of Phemerol, the 1 to 1,000 dilution produced hemolysis.

Averages were calculated for the agglutination times of the cell specimens examined. In the control series there was very little variation among the different specimens of blood cells tested—agglutination first appeared in about thirty seconds and a 4+ reaction was observed in sixty to ninety seconds. In the tests with Benadryl, however, a great deal of variation occurred: In the agglutination of Group A cells with anti-A serum, the average agglutination time of 318 seconds represents a range of from 60 seconds to 630 seconds; likewise in the agglutination of Group B cells with anti-B serum, the average agglutination time of 372 seconds represents a range of 90 seconds to 810 seconds. Benadryl appears to have a greater inhibiting effect on agglutination with some erythrocytes than with others when inhibition is induced by adding it directly to the red cell suspension prior to mixing with the hyperimmune serum. In the tests in which the Benadryl-cell-suspension mixtures were allowed to incubate for one or two hours before mixing them with anti-A or anti-B serum, the cells, in many instances, became hemolyzed so that the mixtures could not be tested.

Three different specimens of Group AB blood were treated with Benadryl and then mixed with anti-A and anti-B hyperimmune sera. The results of these tests were similar to those recorded in Table III.

TABLE IV. THE REACTION TIME OF BENADRYL-TREATED ERYTHROCYTE SUSPENSIONS IN THEIR GROUP SPECIFIC (HYPERIMMUNE) ANTI-A AND ANTI-B SERA

| 2% SUSPENSION OF R.B.C. IN SALINE, TREATED WITH: | LENGTH OF INCUBATION OF CELLS IN DILUENT: | GROUP A CELLS VS. ANTI-A SERUM | | | GROUP B CELLS VS. ANTI-B SERUM | | |
|---|---|---|---|---|---|---|---|
| | | N | FIRST APPEARANCE OF AGGLUTINATION (± TO 1+) | APPEARANCE OF LARGE CLUMPS (4+) | N | FIRST APPEARANCE OF AGGLUTINATION (± TO 1+) | APPEARANCE OF LARGE CLUMPS (4+) |
| Saline | no incubation | 22 | 30 sec. | 78 sec. | 11 | 36 sec. | 60 sec. |
| Phemerol 1-10,000 | no incubation | 12 | 36 | 90 | 7 | 54 | 144 |
| Benadryl 1% | no incubation | 22 | 192 | 318 | 11 | 216 | 372 |
| Benadryl 1% | 10 min. | 20 | 174 | 276 | 10 | 246 | 294 |
| Benadryl 1% | 1 hour | 12 | 108 | 180 | 4 | 96 | 180 |
| Benadryl 1% | 2 hours | 3 | 60 | 120 | — | — | — |

In all of the experiments in this section, hyperimmune anti-A and anti-B sera were used. Similar tests were done employing normal sera from Groups O, A, and B individuals in which the anti-A and anti-B agglutinins occurred in normal concentrations (Table V). In these tests the agglutination times were relatively retarded as would be expected in comparison with the faster acting hyperimmune sera. When Benadryl-treated cells were mixed with normal sera, hemolysis was observed in Group A and Group AB cells, but not in Group B cells; when Benadryl-treated cells were mixed with hyperimmune sera, no hemol-

TABLE V. THE REACTION TIME OF BENADRYL-TREATED ERYTHROCYTE SUSPENSIONS IN INCOMPATIBLE NORMAL SERA

| BLOOD GROUP OF ERYTHROCYTES | ISOAGGLUTININS IN SERA | CONTROL: UNTREATED CELLS | | 2% CELL SUSPENSION IN SALINE TREATED WITH BENADRYL (NO INCUBATION PERIOD) | | | |
|---|---|---|---|---|---|---|---|
| | | BEGINNING AGGLUTINATION ± TO 1+ | LARGE CLUMPS 4+ | BEGINNING AGGLUTINATION ± TO 1+ | LARGE CLUMPS 4+ | HEMOLYSIS* APROX. 25% | HEMOLYSIS* 90 TO 100% |
| A | (hyperimmune) anti-A | 30 sec. | 60 sec. | 210 sec. | 270 sec. | no hemol. | no hemol. |
| | (normal)† | | | | | | |
| | O—anti-A | 90 | 150 | no aggl. | no aggl. | 210 sec. | 450 sec. |
| | O—anti-A | 120 | 240 | no aggl. | no aggl. | 210 | 270 |
| | B—anti-A | 120 | 180 | no aggl. | no aggl. | 210 | 300 |
| B | (hyperimmune) anti-B | 30 | 60 | 90 | 150 | no hemol. | no hemol. |
| | (normal) | | | | | | |
| | O—anti-B | 90 | 120 | 600 | 810 | no hemol. | no hemol. |
| | O—anti-B | 150 | 270 | 300 | 600 | no hemol. | no hemol. |
| | A—anti-B | 150 | 300 | 300 | 660 | no hemol. | no hemol. |
| AB | (hyperimmune) anti-A | 30 | 120 | 120 | 150 | no hemol. | no hemol. |
| | (hyperimmune) anti-B | 30 | 60 | 90 | 120 | no hemol. | no hemol. |
| | (normal) | | | | | | |
| | O—anti-A, B | 150 | 180 | 360 | none | 510 | >720 |
| | O—anti-A, B | 180 | 300 | 330 | none | 330 | 540 |
| | A—anti-B | 150 | 180 | 360 | none | 540 | >720 |
| | B—anti-A | 210 | 300 | 300 | 480 | no hemol. | no hemol. |

*Regular observations were made for 720 seconds, and no observations were made after 900 seconds.

†Sera from four normal individuals were used in this experiment: Two of blood Group O, one of Group A, and one of Group B.

ysis occurred. In the latter case, large clumps formed and persisted, whereas in the former, agglutination when it occurred was slight, but yet 50 to 100 per cent of the cells of blood groups A and AB became hemolyzed.

In the tests in which hyperimmune anti-A and anti-B sera were treated initially with antihistaminics, a greater and more prolonged inhibition of agglutination was obtained (Table II) than was observed when the cell suspensions were similarly treated (Table IV). This difference may have been due to the fact that greater amounts of the antihistaminic agents could be used with the hyperimmune sera than with the erythrocyte suspensions.

D. *The Effect of Antihistaminic Agents on the Anti-Human-Globulin (Coombs') Reaction.*—The sensitized erythrocytes from a newborn infant suffering from hemolytic disease (erythroblastosis fetalis) were used to determine the effect of antihistaminics on the Coombs' reaction. The routine Coombs' test on the erythrocytes of the infant showed a strong agglutination (4+) in thirty minutes at room temperature. To detect the effect of the various antihistaminic agents, 0.05 ml. of each agent, respectively, was mixed with 0.05 ml. of anti-human-globulin serum in small test tubes, and to each was added 0.10 ml. of a 2 per cent suspension of washed sensitized erythrocytes. The specimens were incubated at room temperature and were then examined for agglutination at thirty minutes, one hour, and two hours, respectively (Table VI).

TABLE VI. THE EFFECT OF ANTIHISTAMINIC AGENTS ON THE COOMBS' REACTION

| COOMBS' SERUM DILUTED WITH EQUAL PARTS OF: | LENGTH OF INCUBATION OF SENSITIZED CELLS AND COOMBS' SERUM: | | | | | |
|---|---|---|---|---|---|---|
| | 30 MINUTES | | 1 HOUR | | 2 HOURS | |
| | AGGLUTINATION | HEMOLYSIS | AGGLUTINATION | HEMOLYSIS | AGGLUTINATION | HEMOLYSIS |
| 0.9% saline | 4+ | no hemol. | 4+ | no hemol. | 4+ | no hemol. |
| Phemerol 1-10,000 | 4+ | no hemol. | 4+ | no hemol. | 4+ | no hemol. |
| Neo-antergan (Pyranisamine maleate) | no aggl. | no hemol. | no aggl. | 25% | no aggl. | 50% |
| Pyribenzamine | no aggl. | no hemol. | no aggl. | 50% | no aggl. | 50% |
| Dramamine | ± | no hemol. | ± | no hemol. | ± | 50% |
| Clopane hydrochloride | ± | no hemol. | 2+ | no hemol. | 4+ | no hemol. |
| Neosynephyrine thenfadil | ± | no hemol. | 3+ | no hemol. | 4+ | no hemol. |
| Histadyl | – | 95% | – | 100% | – | 100% |
| Antistine | – | 95% | – | 100% | – | 100% |
| Benadryl | – | 100% | – | 100% | – | 100% |

Pyranisamine maleate and Pyribenzamine delayed or inhibited agglutination of sensitized cells by the Coombs' serum; however, after a period of two hours, 50 per cent of the cells were hemolyzed and so no conclusions could be drawn as to whether the action of the antihistaminic agent was initially to delay agglutination or to inhibit it. Hemolysis occurred within thirty minutes with Benadryl, Phemerol, Histadyl, and Antistine, and, in these instances, the inhibiting effect could not be evaluated. Tests with Neosynephrine thenfadil, Clopane hydrochloride, and Dramamine showed only a trace of agglutination after thirty minutes; at the end of two hours, the Coombs' test was 4+ with Neosynephrine thenfadil and Clopane hydrochloride, and ± with Dramamine.

## DISCUSSION

The use of Benadryl and Pyribenzamine in vivo appears to protect the recipient more effectively, as determined by clinical criteria, than can be accounted for by the reaction as observed in vitro. The routine use of Benadryl and Pyribenzamine in transfusions given to leukemic patients has markedly lowered the incidence of unfavorable reactions and this certainly is of importance in those who require frequent and multiple transfusions. Antihistaminic agents, in vitro, are capable of retarding the speed with which isoagglutination takes place. In some instances rouleaux occurred preceding the formation of the cell aggregates typical of isoagglutination. In most of the cell specimens examined, the smaller clumps (1+ to 3+ in size) of antihistaminic treated cells in type specific antibody appeared to be less firmly held together than the characteristic agglutinations seen in the controls. Antihistaminic agents, in vitro, act to retard and weaken the isoagglutination reaction, but they do not prevent the eventual clumping of the cells; while in vivo, the retardation of agglutination is apparently sufficient to overcome minor clumping due to atypical antibodies and thus prevent the usual and characteristic clinical responses of this reaction.

Mechanisms which may explain this phenomenon are: (1) the effect of possible changes in the pH of the cell suspensions; (2) the surfaces of the erythrocytes may be temporarily affected by the antihistaminics so as to hinder or weaken the antigen-antibody bond; and (3) the activity of the isoagglutinins may be lessened because of physicochemical interference from a combining group in the Benadryl molecule which would temporarily lessen the affinity of the agglutinin for its specific antigen.

The pH of the Benadryl used in these experiments was about 5.2, but when it was added to a cell suspension, the pH of the mixture was consistently around 7.35, and this is within a favorable pH range for isoagglutination reactions. Apparently the erythrocyte suspensions thus treated maintain their normal pH values at room temperature in the presence of Benadryl, at least for the first thirty minutes to one hour after the addition of Benadryl. When antihistaminic agents are added to the hyperimmune anti-A or anti-B sera prior to mixing the sera and cell suspensions, the reaction of retardation of agglutination appeared to be more prolonged than when the cells were first treated with the antihistaminic. This might be interpreted as evidence that the effect is primarily on the antibodies rather than on the cell surfaces; however, these were preliminary experiments and no definite conclusions can be drawn from them.

## SUMMARY

The addition of Benadryl directly to blood to be transfused appears to lessen the occurrence of minor transfusion reactions. This is in agreement with previous observations made by other investigators.

Of eight antihistaminic agents tested in vitro, Benadryl and Pyranisamine maleate appeared to have the greatest inhibitory effect on isoagglutination; Histadyl and Pyribenzamine showed slight inhibitory activity; and essentially no effect was observed with Clopane hydrochloride, Antistine, Neosynephrine theniadil, and Dramamine.

In regard to the Coombs' test, agglutination of sensitized red blood corpuscles by anti-human-globulin serum, the reaction appeared to be retarded by Pyranisamine maleate, Pyribenzamine, and Dramamine; only slight retardation occurred with Clopane hydrochloride and Neosynephrine thenfadil. The effect on the Coombs' test of Histadyl, Antistine, and Benadryl could not be evaluated because these agents produced hemolysis of the sensitized erythrocytes.

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# THE EFFECT OF A BRAIN FRACTION ON SERUM CHOLESTEROL AND THE SERUM LIPID PATTERN

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DOREE and Gardner[1] in 1908 demonstrated that a dog on a diet of meat and vegetables excreted large amounts of cholesterol in the feces; however, when brain was added to the diet, the fecal steroids consisted mainly of coprosterol. Rosenheim and Webster[2] further found that rats on a sterol-free diet excreted only traces of coprosterol and that when cooked brain, itself rich in cholesterol, was also added, large amounts of coprosterol were found in the feces with a diminution of cholesterol. They concluded that this specific action on cholesterol metabolism was largely due to the cerebroside, phrenosin. Rats on a high cholesterol diet, with the addition of adequate phrenosin or cholesterol-free brain extracts, excreted coprosterol to the extent of 70-90 per cent of the fecal steroids.

The body cholesterol can be thought of as being both exogenous, from ingested cholesterol, and endogenous, from synthesis in the liver. Recent work suggests that the endogenous cholesterol, as well as the exogenous, eventually finds its way into the intestines via the biliary tract[3] or direct mucosal transfer.[4] It then would seem to follow that, if the intestinal cholesterol could be converted to the unabsorbable coprosterol in the alimentary canal, a substantial portion of the body's cholesterol could be removed via the gastrointestinal tract. The present experiment was an effort to establish that the feeding of a brain extract will actually reduce the level of serum cholesterol, presumably by this mechanism.

## MATERIALS AND METHODS

Experimentally, 13.6 kg. of sheep brains were homogenized in a Waring blender and extracted at room temperature for periods of twelve hours each as follows:

(a) 8 extractions with 10 liters acetone;
(b) 4 extractions with 5 liters petroleum ether;
(c) 1 extraction with 10 liters acetone.

Following each extraction the solvent was removed by filtration in Buchner funnels. The final residue was spread on trays and dried at room temperature for twenty-four hours. Light tan powder, 1.9 kg., was obtained which was still shown on Soxhlet extraction to contain 33 per cent petroleum ether-soluble material, but only 0.312 per cent cholesterol.

Twenty-four 8-week-old white leghorn cockerels were obtained from a commercial breeder and divided into four groups. The chickens were weighed and fasting blood was

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drawn for an initial total serum cholesterol determination. They were then placed on the diets listed in Table I. The starter mash is a commercial product and upon analysis was shown to contain 2.1 per cent lipid material, but only 0.033 per cent of cholesterol. Water was allowed ad libitum. The cockerels were kept on these diets for fourteen days, with an average weight gain of 146 Gm. per bird, which was approximately equal in the four groups. Following a twelve-hour fast, the birds were exsanguinated via the external jugular vein and serum lipids determined. Total lipid and total cholesterol were determined by a modification of the Bloor technique,[5] phospholipids by a modified Fiske-Subbarow method,[6] and the ultracentrifuge pattern was studied using the heavy-density ($\rho = 1.21$) NaCl-KBr medium as described by Lewis and Green.[7, 8]

## RESULTS

Table I presents our findings. Four birds were ultimately excluded from Groups I and II because of intercurrent disease. There was a definite tendency for serum cholesterol to be reduced by the addition of brain powder to the

TABLE I. THE FINAL SERUM LIPID VALUES AND INITIAL SERUM CHOLESTEROL LEVELS IN THE FOUR DIETARY GROUPS

| GROUP | CHICK NO. | DIET | GM./DAY OFFERED PER CHICKEN | SERUM CHOLESTEROL MG. % AT TIME OF SACRIFICE | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | PREDIETARY | FINAL | CHANGE | TOTAL LIPID MG. % | PHOS. LIPID | CHOL./LP |
| I | 2 | Starter mash | 100 | 125 | 116 | −9 | 358 | 173 | 16.8 |
| | 4 | | | 140 | 130 | −10 | 380 | 190 | 17.1 |
| | 5 | | | 126 | 127 | +1 | 350 | 193 | 16.5 |
| | 6 | | | 106 | 104 | −2 | 453 | 115 | 22.6 |
| II | 7 | Starter mash and | 100 | 112 | 98 | −14 | 375 | 140 | 17.5 |
| | 8 | brain powder | 12 | 133 | 97 | −36 | 375 | 158 | 15.4 |
| | 9 | | | 132 | 98 | −34 | 373 | 138 | 17.8 |
| | 11 | | | 118 | 121 | +3 | 403 | 138 | 22.0 |
| III | 13 | Starter mash, | 100 | 197 | 421 | +224 | 850 | 223 | 47.3 |
| | 14 | cottonseed oil | 10 | 130 | 652 | +522 | 1310 | 240 | 67.9 |
| | 15 | and cholesterol | 2 | 126 | 801 | +675 | 1608 | 335 | 59.8 |
| | 16 | | | 144 | 817 | +673 | 1700 | 318 | 64.3 |
| | 17 | | | 149 | 483 | +334 | 1150 | 275 | 43.9 |
| | 18 | | | 152 | 1025 | +873 | 2043 | 365 | 70.1 |
| IV | 19 | Starter mash, | 100 | 153 | 234 | +81 | 535 | 108 | 54.4 |
| | 20 | cottonseed oil, | 10 | 163 | 385 | +222 | 915 | 215 | 44.8 |
| | 21 | cholesterol, and | 2 | 131 | 265 | +134 | 630 | 173 | 39.0 |
| | 22 | brain powder | 12 | 137 | 187 | +50 | 563 | 143 | 32.8 |
| | 23 | | | 114 | 387 | +273 | 878 | 160 | 60.5 |
| | 24 | | | 134 | 362 | +228 | 898 | 188 | 48.3 |

control diet. Three of these four chicks in Group II had cholesterol levels lower than were seen in any of the 24 chicks prior to the onset of dietary manipulation, and earlier pilot experiments gave comparable results. In the groups with added cholesterol in oil (Groups III and IV), there is a striking difference, with no overlap, between the levels of serum cholesterol and total lipid in the control group (Group III) and the treated group (Group IV) (Fig. 1). It can be seen from the table that the total lipids and the phospholipids correspond with the diet and vary roughly with the cholesterol, but not quite so much. Hence the cholesterol/lipid phosphorus ratio, to which atherogenic significance has been ascribed, tends to be reduced.

The ultracentrifuge patterns showed equally striking differences between Groups III and IV as illustrated in Fig. 2. Feeding of 2 per cent cholesterol to these chicks caused a large peak of beta lipoprotein material to develop in the range $S_f$ 20-70, which corresponds to the range of $S_f$ 6-20 with Gofman's system.[8] This apparently included macromolecules lighter than the usual beta lipoprotein, there being little or no material at the usual $S_f$ 30-35, the peak concentration gradient occurring at an $S_f$ of 50. The addition of brain powder to the diet caused a reduction of this abnormal lipoprotein by approximately

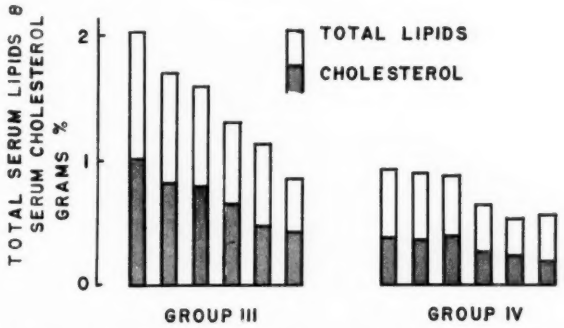


Fig. 1.—Comparison between the total serum lipids of the cholesterol-fed chicks (Group III) and the cholesterol-fed chicks receiving brain powder (Group IV). The shaded portion of the bar represents the cholesterol fraction in the same serum.

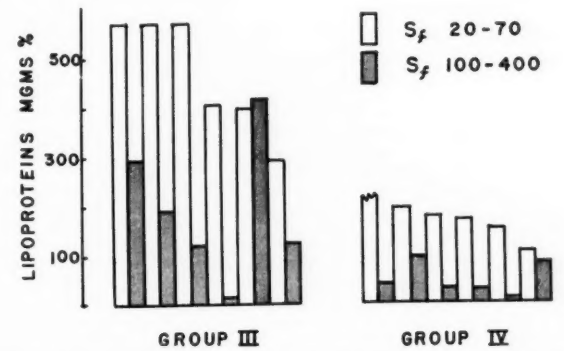


Fig. 2.—Comparison between chicks receiving cholesterol plus brain powder (Group IV) with the cholesterol-fed controls (Group III) with regard to the beta lipoprotein ($S_f$ 20-70) concentration and lower density lipoproteins ($S_f$ 100-400) which are shaded.

buff, there being no overlap between the two groups. There was also a reduction in the excessive amount of low-density lipoprotein of $S_f$ 100-400 (Gofman's S$_f$ 35-140), and a questionably significant reduction in the alpha lipoprotein. The differences between Groups I and II in ultracentrifuge patterns were insignificant, though they also suggested a reduction in the amount of material in the $S_f$ 40-70 category.

## CONCLUSION

These findings are in accord with the original hypothesis and suggest a simple attack against hypercholesterolemia of exogenous and, perhaps, en-

dogenous origin by the supplementary feeding of an unidentified fraction of cholesterol-extracted crude brain. Further work is in progress to see whether the cerebroside fraction indeed contains the active principle, to investigate the effects of increasing dosage, and to determine whether the atherosclerosis of cholesterol feeding can in fact be prevented by sustained activity of such a brain fraction.

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# THE BENEFICIAL EFFECTS OF INTERMITTENT DIETARY REGIMES ON THE TENDENCY TO ATHEROSCLEROSIS

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THE presence of cholesterol in the diet of rabbits,[1] chicks,[2] dogs,[3] and perhaps of man results, at appropriate age periods, in the development of atherosclerotic lesions in the aorta and in the coronary arteries.[4] We have recently demonstrated in the chick that this effect is independent of total caloric intake, as such, but depends primarily on the ratio, ingested cholesterol: body weight.[5] These experiments suggested that the tendency to hypercholesteremia and to atheromatosis might also be affected by the *pattern* of cholesterol ingestion and the nature of the remainder of the diet. We therefore undertook studies on the effect of intermittent feeding of cholesterol diets with varied regimens during the periods when the animal did not receive cholesterol.

## GENERAL METHODS

Two series consisting of a total of 250 newly hatched Hyline cockerels were purchased from a local hatchery. Weekly records of food intake and of rate of growth were made. The animals were bled at intervals to determine the plasma cholesterol level.[6]

The experiments were designed to test the effect of intermittent cholesterol feeding and the influence of the diet during the intermittent cholesterol-free periods. In the first series cholesterol was added to the mash on alternate days. In the second series the periods were extended to five days, as described below.

Groups of animals were sacrificed at intervals and examined according to previously described techniques.[7] The heart and aorta were dissected en bloc and inspected for gross atherosclerosis. These tissues were then treated with Sudan IV to manifest occult fatty infiltrations. Two or more independent observers graded the aortas as unknowns. Grading of lesions was on the basis of their area, elevation and number; values up to plus 4 (severe and extensive) were assigned. Frozen and paraffin sections of representative lesions of the heart and aorta were then stained with Sudan IV and with hematoxylin and eosin, respectively.

In the present study attention is given primarily to *thoracic* atheroma, since these are cholesterol induced.

## RESULTS

1. *Series One: Daily Alternation.*—This series of 73 chicks was divided into four groups. The accumulated data are summarized in Tables I and II.

*Group 1* consisted of twenty-two animals on a normal mash diet every day. These animals gained weight rapidly, weighing an average of 2 kilograms at twenty-six weeks. The average plasma cholesterol varied from 56 to 113 mg.

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TABLE I. A COMPARISON OF THE ATHEROGENIC EFFECTS OF CONTINUOUS VERSUS INTERMITTENT FEEDING OF CHOLESTEROL. SERIES I

| GROUP | DIET | AT 20 WEEKS ON DIET | | AT 26 WEEKS ON DIET | |
|---|---|---|---|---|---|
| | | INCIDENCE OF LESIONS* | AVERAGE GRADE OF LESIONS | INCIDENCE OF LESIONS* | AVERAGE GRADE OF LESIONS |
| 1 | Normal mash | 0/10 | 0 | 0/12 | 0 |
| 2 | Mash plus 2% cholesterol | 8/11 | 2 | 4/5 | 1 |
| 3 | Mash plus 2% cholesterol alternated with fasting | 8/8 | 3 | 6/6 | 2 |
| 4 | Mash plus 2% cholesterol alternated with normal mash | 4/12 | ½ | 6/9 | ½ |

*Numerator represents number of chicks with lesions; denominator represents total chicks in group.

TABLE II. THE AVERAGE PLASMA CHOLESTEROL VALUES (MG. %) IN CHICKS ON VARIOUS DIETARY REGIMENS. SERIES I

| GROUP | DIET | AGE AND WEEKS ON DIET | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 6 | 7 | 8 | 9 | 10 | 12 | 15 | 20 | 26 |
| 1 | Normal mash | 56 | | 85 | 96 | 112 | 113 | 85 | 88 | 95 |
| 2 | Mash plus 2% cholesterol | 162 | 212 | 235 | 354 | 365 | 256 | 434 | 334 | 230 |
| 3 | Mash plus 2% cholesterol alternated daily with fasting | 165* | 293 | 184* | 391 | 349* | 419 | 724* | 483 | 340* |
| 4 | Mash plus 2% cholesterol alternated daily with normal mash | 96† | 98 | 169† | 229 | 162† | 266 | 146† | 190 | 144† |

*Bloods drawn while animals were fasting.
†Bloods drawn while animals were on normal mash regime.

per cent over the course of the twenty-six weeks on the diet. Autopsy at twenty or twenty-six weeks revealed thoracic atherosclerosis in none of the chicks.

*Group 2* consisted of sixteen chicks which received the same daily diet as that given to Group 1, except that 2 per cent commercial crystalline cholesterol was mixed in with the mash. No oil was used. Weight gain was as in Group 1. The average weekly plasma cholesterol values ranged from 162 to 434 mg. per cent. At twenty weeks on the diet eleven chicks were sacrificed. Eight of these eleven animals had thoracic lesions with an average grade of +2. At twenty-six weeks, the remaining five chicks were sacrificed; four had lesions with an average grade of +1.

*Group 3* consisted of fourteen chicks receiving the 2 per cent cholesterol-enriched diet on odd days. On even days the feed trays were removed and the animals had no food. These animals gained weight at about 75 per cent of the rate of the animals receiving an ad libitum diet. The plasma cholesterol level on days of cholesterol feeding was in general of the same order as that of chicks receiving the cholesterol mash diet every day.

All fourteen chicks had thoracic atherosclerosis at the end of the experiment. The eight sacrificed at twenty weeks had an average grading of 3, while the six sacrificed at twenty-six weeks had a grading of 2. These animals are seen to have a higher incidence and degree of atherosclerosis than those receiving the diet every day (Group 2), despite the fact that less total cholesterol was ingested. The alternately fasted group ate only slightly more

than half the food taken by the other groups. Thus these birds did not make up for the fast on their feeding days.

*Group 4* consisted of twenty-one chicks. These animals received the 2 per cent cholesterol-mash diet on odd days; on the even days they were given a normal mash diet without the cholesterol supplement. Weight gain was as in Groups 1 and 2. The plasma samples drawn on the day when the chicks were on the cholesterol-mash diet averaged 196 mg. per cent, definitely lower than Groups 2 and 3. The samples drawn on the days when the chicks were on normal mash were even lower, being about 143 mg. per cent. At twenty weeks only four of the twelve chicks sacrificed had lesions; these averaged one-half. At twenty-six weeks, six of the nine chicks sacrificed had lesions, these also averaged one-half.

2. *Series Two: Intermittent Feeding.*—On arrival in the laboratory, the chicks in this series were divided into six groups, numbered five to ten and immediately placed upon the experimental regimens indicated below. Just prior to the time of the ten-week sacrifice, a failure in the thermostatic regulation of the animal room resulted in the death of a large number of these animals. The remaining seventy-four chicks were sacrificed at the end of ten or fifteen weeks on the various regimes. Data are summarized in Tables III and IV.

*Group 5,* consisting of thirteen control chicks, was given an unrestricted normal mash diet, without supplement, every day. Results were identical with Group 1.

*Group 6,* consisting of twenty-three chicks, was given an unrestricted diet of a 2 per cent cholesterol-enriched mash, plus 5 per cent oil every day. The plasma cholesterol level averaged about 1,000 mg. per cent at five weeks, 900 at ten weeks, and 2,000 at fifteen weeks. At ten weeks, nineteen of twenty sacrificed animals had thoracic aortic atherosclerosis, with an average grade of 1. At fifteen weeks the remaining three chicks had lesions graded at 3½.

*Group 7:* Because the remaining groups were to receive a 2 per cent cholesterol-enriched diet only half of the time, it was felt that comparison with a group of chicks receiving a 1 per cent cholesterol-enriched diet every day would aid in analysis of the results. This group, consisting of eleven chicks, was therefore placed on a continuous mash diet, supplemented with 1 per cent cholesterol and 5 per cent cottonseed oil. These animals developed a plasma cholesterol averaging 416 mg. per cent at five weeks, 632 at ten weeks, and 671 at fifteen weeks. At the ten-week sacrifice seven of eight birds had lesions, with an average grade of 1½. At fifteen weeks, the three remaining birds had lesions also graded as 1½. These results are comparable with other feeding experiments in which we have fed a similar diet.

*Groups 8, 9, and 10:* The remaining groups were placed on the intermittent regime, arranged as follows: For the first week the chicks received a mash enriched with 2 per cent cholesterol and 5 per cent oil on odd days; on the even days, the chicks received the special regime assigned its group, as given below. During the second week, the chicks received the cholesterol-enriched diet for two days, and then the special regime for two days. These

cycles were prolonged one day each week, until by the fifth week the animals were on the 2 per cent cholesterol-enriched diet for five days and then on the special treatment for five days. This procedure of intermittent five-day periods was maintained for the remainder of the experiment.

*Group 8* consisted of chicks which were *fasted* in the interim periods between periods of cholesterol-oil enriched mash feeding as described above. Most of these animals succumbed during the failure of the room thermostatic control just prior to the ten-week sacrifice. The average plasma cholesterols at the end of five-day cholesterol feeding periods were 405 mg. per cent at five weeks, 961 at ten weeks, and 1,199 at fifteen weeks. By contrast, the cholesterols at the end of the succeeding five days of fasting had fallen to 314, 433, and 641 mg. per cent at six, eleven, and sixteen weeks, respectively. The four chicks sacrificed at fifteen weeks all showed thoracic lesions with an average grade of 1½.

*Group 9* consisted of eleven chicks which received *normal mash* for the interim periods, between the periods of cholesterol-oil enriched mash feeding, as described above. The plasma cholesterols at the end of a five-day period on the 2 per cent cholesterol diet averaged 490 mg. per cent at five weeks, and 270 at ten and at fifteen weeks. At the end of a five-day period on normal mash, the average cholesterol values were about 120 at six and eleven weeks, and 95 at sixteen weeks. Three of four chicks sacrificed at fifteen weeks, at the end of a period on the cholesterol enriched mash, had lesions averaging one-half. Three birds placed on normal mash for another five-day period and then sacrificed, showed no thoracic lesions. Three remaining birds continued on normal mash for two more weeks also had no thoracic lesions.

These findings have been confirmed in subsequent experiments designed differently but bearing on the same problem. Since these newer observations were aimed at further development of the subject they will be reported at a later date.

*Group 10* consisted of twelve chicks which received the 2 per cent cholesterol plus 5 per cent oil mash diet for alternate five-day periods. During the interim periods these birds received a *normal mash diet to which 20 per cent cottonseed oil* had been added. The plasma cholesterol at the end of a five-day period on the 2 per cent cholesterol diet was about 350 at five weeks; it was about 660 at ten and fifteen weeks. At the end of a five-day period on the normal mash plus 20 per cent oil supplement, the corresponding average plasma cholesterols had fallen to about half these values. Sacrifice at fifteen weeks, at the end of a five-day period on the cholesterol diet, revealed lesions in all five chicks with an average grade of 2. At the end of the subsequent five days on the 20 per cent oil diet, lesions were seen in all six sacrificed, and these were graded as 1½.

3. *Coronary Lesions.*—Frozen sections stained with Sudan IV were made from the hearts of 28 chicks distributed among series two. These showed essentially the same degree of atheromatosis as that found in the corresponding aortas.

TABLE III. THE INCIDENCE AND SEVERITY OF THORACIC ATHEROMATOSIS IN CHICKS ON CONTINUOUS OR INTERMITTENT DIETS. SERIES II

| GROUP | DIET | STATUS | AT 10 WEEKS ON DIET | | AT 15 WEEKS ON DIET | | |
|---|---|---|---|---|---|---|---|
| | | | INCIDENCE OF LESIONS* | AVERAGE GRADE OF LESIONS | INCIDENCE OF LESIONS | AVERAGE GRADE OF LESIONS | INDIVIDUAL GRADE OF LESIONS |
| 5 | Normal mash continuously | | 0/8 | 0 | 0/5 | 0 | |
| 6 | Mash plus 2% cholesterol plus 5% oil continuously | | 19/20 | 1 | 3/3 | 3½ | 3, 3½, 3½ |
| 7 | Mash plus 1% cholesterol plus 5% oil continuously | | 7/8 | 1½ | 3/3 | 1½ | 2, 2, ¾ |
| 8 | Mash plus 2% cholesterol plus 5% oil alternated with fasting | loaded† 5 days | | | 4/4 | 1½ | 1½, 1½, 1½, 1 |
| 9 | Mash plus 2% cholesterol plus 5% oil alternated with normal mash | loaded† 5 days | | | 3/4 | ½ | ¼, 0, ¼, ¾ |
| | | unloaded‡ 5 days | | | 0/3 | 0 | 0, 0, 0 |
| | | unloaded§ 28 days | | | 0/3 | 0 | 0, 0, 0 |
| 10 | Mash plus 2% cholesterol plus 5% oil | loaded† 5 days | | | 5/5 | 2 | 2, 2, 2, 1½, 1½ |
| | Alternated with mash plus 20% oil | unloaded‡ 5 days | | | 6/6 | 1½ | 2, ½, 2½, 1½, ½, 2 |

*Numerator represents number of chicks with lesions; denominator represents total number of chicks in group.
†Loaded = sacrificed at end of one of the five-day periods on the cholesterol diet.
‡Unloaded = sacrificed at end of one of the five-day periods off the cholesterol diet.
§Unloaded = sacrificed at end of a twenty-eight-day period off the cholesterol diet.

TABLE IV. THE PLASMA CHOLESTEROL VALUES (MG. PER CENT) IN CHICKS ON CONTINUOUS OR INTERMITTENT DIETS. SERIES II

| GROUP | DIET | STATUS | AGE AND WEEKS ON DIET | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 5 | 6 | 10 | 11 | 15 | 16 |
| 5 | Normal mash | | 106 (81-131) | | 102 (87-114) | | | |
| 6 | Mash plus 2% cholesterol plus 5% oil | | 1008 (690-1820) | | 896 (650-1285) | | 2017 (1579-2288) | |
| 7 | Mash plus 1% cholesterol plus 5% oil | | 416 (236-785) | | 632 (280-1163) | | 671 (302-1111) | |
| 8 | Mash plus 2% cholesterol plus 5% oil | loaded* 5 days | 405 (228-700) | | 961 (517-1433) | | 1199 (744-2080) | |
| | Alternated with fasting | unloaded† 5 days | | 314 (224-533) | | 433 (315-572) | | 641 (318-964) |
| 9 | Mash plus 2% cholesterol plus 5% oil | loaded* 5 days | 490 (256-740) | | 270 (188-364) | | 267 (215-358) | |
| | Alternated with normal mash | unloaded† 5 days | | 136 (127-150) | | 120 (108-146) | | 95 (71-114) |
| 10 | Mash plus 2% cholesterol plus 5% oil | loaded* 5 days | 352 (62-1128) | | 669 (390-1125) | | 654 (352-986) | |
| | Alternated with mash plus 20% oil | unloaded† 5 days | | 149 (107-239) | | 303 (160-450) | | 215 (150-284) |

*Loaded = determinations made at end of a five-day period on the cholesterol diet.
†Unloaded = determinations made at end of a five-day period off the cholesterol diet.

## DISCUSSION

The experiments utilizing intermittent dietary regimes provide a basis for the concept that in the chick the pattern of cholesterol ingestion plays an important role in the tendency to increased plasma cholesterol levels and to atheromatosis (Tables I to IV). They also suggest that the nature of the dietary intake in the interim periods may play an important role in the ability to unload cholesterol from the blood and tissues.

The alternately fasted chicks ate less cholesterol per kilogram of body weight than did the animals on the full diet, yet these fasted birds had a higher degree of cholesteremia and a greater degree of lesions than did those on an ad libitum diet. These results suggest that an ample diet serves in some way to offer a degree of protection against hypercholesteremia and atheromatosis in the chick, even though cholesterol is present in the food. The composition of the diet during the intermittent periods is also of importance. This is demonstrated by the fact that the animals on the intermittent normal mash diet showed the greatest reduction in plasma cholesterol levels, and in a marked tendency for regression of lesions.

The results given above demonstrate that the feeding of cholesterol results, at appropriate age periods,[8] in a rapid rise in the plasma cholesterol level in the chick. In the course of a few days on the cholesterized regime the plasma values may be increased by fivefold. The ability to unload cholesterol from the plasma is also rapid. In the course of a few days on a normal diet the plasma level may return almost to normal. The rate of this fall in plasma cholesterol is strikingly retarded if the animal is given no food or if it is given a diet with a high oil concentration.

These data show that plasma cholesterol regulation is very active in the chick, with the plasma level responding within a matter of days after a change in the pattern of the diet. Similar data on the thiouracil treated dog show that cessation of a cholesterol rich diet is followed within the course of a few days by a rapid fall of plasma cholesterol and lipid phosphorus.[3] This regulation probably involves the rate of endogenous synthesis of cholesterol.[9] The finding that starvation or a diet rich in oil may reduce the rate of unloading of plasma cholesterol also points to a possible role of the gastrointestinal tract not only in the absorption but also in the elimination of cholesterol and its by-products. The enteric tract has also been implicated in our studies on the addition of aluminum hydroxide gels[11] or plant sterols to the diet. These substances, although not absorbed, both have the effect of reducing the plasma cholesterol and the atherosclerotic lesions in chicks fed cholesterol-enriched diets (unpublished data).

The tendency to atheromatosis closely parallels the plasma cholesterol level. When the plasma level rises, the development of aortic and coronary lesions proceeds rapidly, and in the course of a few days gross aortic lesions may be seen. The presence of atheromatosis in chicks after a five-day period on the cholesterolized diet supports this view. The present experiments demonstrate further that the atheromatous lesions, at least in their early form,

may regress rapidly. The fact that the aorta was free of atheromatosis in similar chicks permitted a five-day respite from the addition of cholesterol to the diet suggests that the lesions may be rapidly reversible. Earlier studies have shown that continuous feeding for long periods may lead to less rapid reversibility in some instances.[10]

The present data also enforce the concept that the *age period* of the animal is of importance in the development of hypercholesteremia and of atheromatosis.[8] We have previously shown that at various periods in the life of the chick, and probably of other animals including man, there is a variability, depending on age, in the tendency to atherogenesis. The data in the present study support this contention. This is seen in the fact that the chicks at twenty weeks of age showed more severe lesions than at twenty-five weeks of age on the same diet.

Other data[12] demonstrate that the ability to absorb cholesterol is limited, since dietary cholesterol in proportions of 20 or 50 per cent of the diet produces no more severe hypercholesteremia or atheromatosis than does a diet containing 4 per cent cholesterol. This extends earlier work of the department.[7] The previous conclusion that the tendency to atheromatosis is dependent upon the total cholesterol intake per kilogram of body weight[7] must therefore be modified by our present findings.

Our data suggest that the hypercholesteremia and atheromatosis attendant upon the uninterrupted ingestion of cholesterol has some characteristics consonant with storage disorders. Incessant ingestion of cholesterol would therefore appear to inhibit unloading of the lipid, and thereby facilitate the development of atherosclerosis. Frequent interruptions of this continuous regime would appear to facilitate the unloading of the accumulated stores from various organs, including the intima of the blood vessels with a regression of atheromatosis.

## SUMMARY

The effect of intermittency of diet on the tendency to hypercholesteremia and atheromatosis was studied in chicks.

Continuous feeding of a diet supplemented with cholesterol resulted in an elevated plasma cholesterol level, and in the production of aortic and coronary atheromatosis. The diet of comparable chicks was arranged so that an equivalent amount of cholesterol was ingested in interrupted periods. The plasma cholesterol rose rapidly during these periods of cholesterol feeding and animals sacrificed at the end of these periods showed moderate development of atheromatosis. During the interval on normal diets, rapid reductions in plasma cholesterol were observed; animals sacrificed at the end of this period had little or no atheromatosis. Chicks which were fasted or which were given an oil-rich diet during the intermittent period showed only a slight tendency for a fall in plasma cholesterol, and little or no protection against atheromatosis.

These experiments suggest that in the chick intermittent periods of freedom from cholesterol feeding may offer a degree of protection against the atherogenic effects of high cholesterol diets.

We are indebted to Marilyn Dudley, B.S., Phillip Johnson, and Grady Crowley for technical assistance in the conduct of these experiments.

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# NITROGEN METABOLISM AND LIVER HISTOLOGY DURING AUREOMYCIN ADMINISTRATION IN PATIENTS WITH HEPATIC DISEASE

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ALTHOUGH the antibacterial effects of the broad spectrum antimicrobial agents have been well described, the effect of these agents on human nutrition and intermediary metabolism is relatively unknown. Recent investigations have revealed that certain untoward nutritional effects may be associated with the administration of Aureomycin, terramycin, and chloramphenicol. Vitamin deficiency signs and steatorrhea have been noted during the administration of each of these antibiotics,[1-5] and fatty infiltration of the liver has been noted in patients receiving Aureomycin or terramycin by Yesner and Kunkel[6] and by Sborov and Sutherland.[7] Lepper and his colleagues[8] have also reported fatty infiltration during Aureomycin therapy and have demonstrated that Aureomycin and terramycin are capable of producing fatty infiltration in animals regardless of dietary intake.[9] In addition, they observed that such fatty infiltration could be produced by inactivated Aureomycin, thus suggesting that the hepatic effect was not a result of changes in the intestinal flora.

Despite the studies mentioned, beneficial effects of Aureomycin have been reported in patients with chronic hepatitis.[10] In view of the apparent contradiction between these results, this investigation was undertaken in an attempt to correlate the hepatic and nutritional effects of the administration of Aureomycin to patients with liver disease. In addition, since it has been suggested that fatty infiltration of the liver during Aureomycin administration may be due to induced deficiency of or blockade in the utilization of sulfhydryl compounds,[7] the effects of supplements of methionine during antibiotic therapy were investigated. During the course of these studies, Gabuzda and his colleagues reported the occurrence of negative nitrogen balance and increased riboflavin excretion following the administration of Aureomycin to undernourished individuals.[11] In their study, stool cultures revealed no consistent qualitative or quantitative changes in the gastrointestinal flora which correlated with the metabolic effects noted.

## MATERIAL AND METHODS

Three patients with early fatty cirrhosis of the liver and one patient with acute hepatitis were studied during the administration of Aureomycin.* The diagnoses on these

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*Aureomycin with methyl and propyl paraben obtained through the courtesy of Dr. Stanton M. Hardy, Lederle Laboratories.

patients were established by clinical course, liver function tests, and liver biopsy. The approximate protein and caloric content of the diets eaten by two of the patients was calculated, but no serious attempt was made to maintain constant food intakes. In these patients urine nitrogen excretion alone was determined. On two other patients complete nitrogen balance studies were carried out while they were receiving constant diets. The dietary intakes and supplements administered to each patient are recorded in Table I. In only one patient, C. W., was the anorexic affect of Aureomycin noted, and this was not sufficiently marked to interfere with the intake of a constant diet. Food variety was provided and intake was controlled by the use of three different diets equal in protein, fat, and calorie content, given in rotation.

TABLE I. DIAGNOSIS, DIETARY INTAKE AND SUPPLEMENTS RECEIVED

| PATIENT | INITIAL WEIGHT LBS. | DIAGNOSIS | DIET | | SUPPLEMENT |
|---|---|---|---|---|---|
| | | | PROTEIN GM. | CALORIES | |
| J. D. | 141 | Alcoholic fatty cirrhosis | 115* | 2,650 | Folic acid 10 mg. and ascorbic acid 300 mg. daily. After fatty infiltration occurred, received methionine 4 Gm., then 8 Gm. daily. |
| W. H. | 161 | Infectious hepatitis | 220* | 5,300 | 3 multivitamin capsules,† and 4 Gm. methionine daily. |
| N. S. | 165 | Alcoholic fatty cirrhosis | 93 | 3,000 | None |
| C. W. | 155 | Alcoholic fatty cirrhosis | 50 | 2,000 | 4 Gm. methionine daily. |

*Calculated average values, approximate maximum daily variation was 15 Gm. of protein and 200 calories.

†Each capsule contained: vitamin A, 5,000 I.U.; vitamin D, 1,000 U.; Thiamin, 2.5 mg.; riboflavin, 2.5 mg.; pyridoxine, 0.5 mg.; niacin amide, 20 mg.; calcium pantothenate, 5 mg.; ascorbic acid, 37.5 mg.

In all patients liver biopsies were performed before, during, and after Aureomycin administration. Daily twenty-four hour urine collections were preserved with toluol and analyzed for urine nitrogen by the macro-Kjeldahl method. Stools were preserved with measured amounts of benzoic acid, and collection periods were marked by the ingestion of carmine red. Stool nitrogen was determined by the Kjeldahl method and stool fat was determined by the method of Fowweather.[12] After a thirty-day control period, patient J. D. with fatty cirrhosis received 3 Gm. of Aureomycin daily for twenty-nine days and was studied for eleven days after the cessation of Aureomycin ingestion. Patient W. H., with acute hepatitis, was followed for eleven days before Aureomycin, then 3 Gm. orally and 300 mg. intravenously daily were given for a five-day period. Urine nitrogen studies were carried out for six days after Aureomycin in this patient. In the other two patients six- and seven-day control periods were followed by six and seven days, respectively, of 2 Gm. Aureomycin daily in divided doses. Metabolic study was continued for four days after Aureomycin in one patient, and for six days in the second patient.

Liver biopsies were obtained with a Vim-Silverman needle using the intercostal approach. Repeated biopsies in each patient were done through the same intercostal space. Thymol turbidity by a modification of Maclagen's method,[13] cephalin flocculation,[14] serum bilirubin,[15] and bromsulfalein retention (forty-five minutes after a 5 mg. per kilogram of weight dose) were determined repeatedly in most patients.

In an attempt to determine changes in adrenocortical function during Aureomycin therapy, daily eosinophil counts[16] and repeated urine "cortin" assays[17] were carried out on one patient.*

## RESULTS

*Nitrogen Metabolism.*—In all patients, urinary nitrogen was markedly increased during the period of Aureomycin administration. Although complete

*Obtained through the courtesy of Dr. Charles W. Lloyd.

balance studies were not carried out in the first two patients, the urine nitrogen during Aureomycin administration appeared to differ significantly from the figures obtained during the periods without Aureomycin (Figs. 1 and 2). In these two patients the nitrogen intake was not controlled and stool nitrogen was not analyzed; therefore, a possible negative nitrogen balance cannot be determined from figures obtained. In the subjects in whom nitrogen and caloric intake were controlled, negative nitrogen balance due to markedly increased urine nitrogen excretion was readily apparent (Figs. 3 and 4).

The first patient, J. D., was studied originally to determine any relationship between fatty infiltration and protein metabolism. Unfortunately, urine

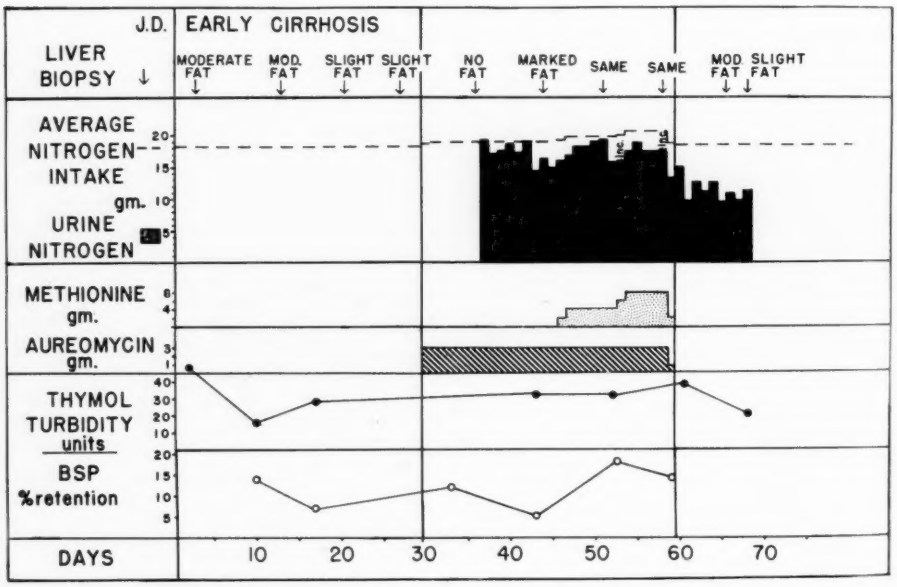


Fig. 1.—Urine nitrogen excretion decrease when Aureomycin was discontinued. Urine nitrogen studies on days 1 to 36 omitted due to incomplete collection. Fatty infiltration of liver during Aureomycin therapy, unaltered by methionine. (Thymol turbidity normal values less than 30 units; inc. = incomplete urine collection.)

nitrogen determinations were marred by incomplete collections until the thirty-sixth day; after this time, however, collections were complete. During Aureomycin administration the urine nitrogen varied from 14.4 to 19.4 Gm., averaging 16.5 Gm. This was unaltered by the addition of methionine in doses of 4 Gm. daily for eight days followed by 8 Gm. a day for six days. After Aureomycin was discontinued the urine nitrogen fell to an average of 11.5 Gm. per day, with a variation from 9.6 to 15.1 Gm. Although the protein and caloric intake was inconstant from day to day, over the two periods the average intake was approximately the same. In view of this the decrease in urine nitrogen after Aureomycin was discontinued is probably a valid observation.

Patient W. H., who was convalescent from acute infectious hepatitis, required an extremely high dietary intake because of a ravenous appetite.

Methionine in doses of 4 Gm. a day was given throughout the study of this patient, and from the eleventh to the sixteenth day he received 3 Gm. of Aureomycin orally plus 300 mg. of Aureomycin intravenously in a single injection on each of the first four days of this period. The average urinary nitrogen excretion during the three-day control period on the maximum dietary intake was 18.3 Gm. and during Aureomycin this rose to 22.6 Gm. The latter figure might have been higher except for an incomplete collection on one day. When Aureomycin was discontinued urine nitrogen fell to 21.8 Gm. daily. In view of the high dietary intake which was maintained and the markedly positive nitrogen balance thus produced, the relatively slight fall in urine nitrogen after Aureomycin was stopped may have been due to a spontaneous return toward nitrogen equilibrium. The increase in urine nitrogen excretion during Aureomycin administration, however, is apparent.

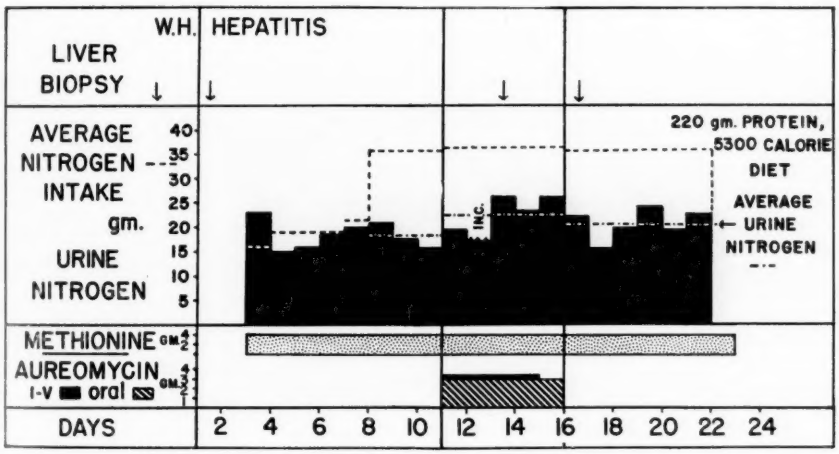


Fig. 2.—Increased urine nitrogen during Aureomycin despite methionine. (inc. = incomplete urine collection.)

Complete nitrogen balance and stool fat studies were then carried out on two patients suffering from alcoholic fatty cirrhosis. Patient N. S. was in slightly positive nitrogen balance during the control period, but after two days of Aureomycin negative balance ensued (Fig. 3). This effect persisted throughout the period of antibiotic administration and positive nitrogen balance returned with the discontinuation of Aureomycin. Stool nitrogen was not altered during this study (Fig. 3), and an insignificant decrease in stool fat occurred throughout the three periods (Table II).

TABLE II. FAT INTAKE AND STOOL FAT DURING AUREOMYCIN ADMINISTRATION (GM. PER DAY)

| | | CONTROL | AUREOMYCIN | POST-AUREOMYCIN |
|---|---|---|---|---|
| N. S. | Intake | 104 | 104 | 105 |
| | Stool | 7.6 | 6.2 | 4.5 |
| C. W. | Intake | 53.2 | 52.1 | 51.1 |
| | Stool | 4.8 | 4.2 | 1.9 |

Patient C. W. received a lower caloric and protein intake than N. S. and was in slightly negative nitrogen balance during the control period followed by a definite increase in nitrogen loss when Aureomycin was given (Fig. 4). Positive nitrogen balance ensued when the antibiotic was discontinued. This patient received methionine throughout the study with little change in the effect of Aureomycin when compared with patient N. S. who received no methionine. In this patient, C. W., stool nitrogen was slightly increased by the administration of antibiotic, but the more marked effect was that of an increase in urine nitrogen excretion. Excretion of fat in the stool was also insignificantly altered in this patient (Table II).

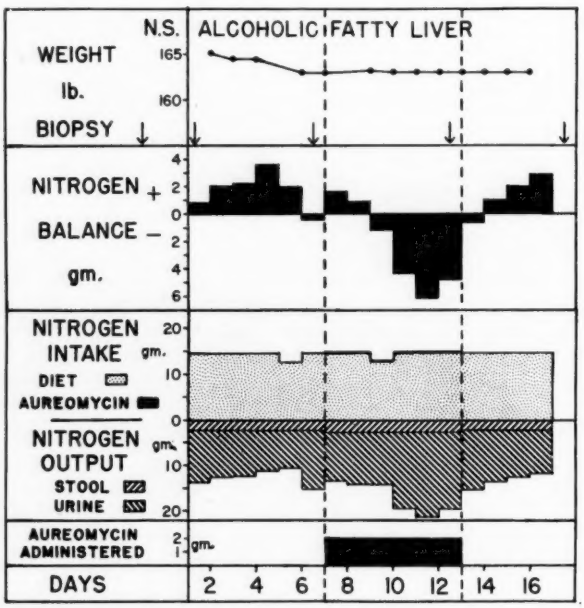


Fig. 3.—Negative nitrogen balance during Aureomycin administration.

*Effect of Aureomycin on Liver Histology and Liver Function Tests.*—Despite the fact that Aureomycin was administered to patients with pre-existing liver disease, in only one patient was fatty infiltration increased during the period of Aureomycin administration. In this subject, J. D., the antibiotic was given for two weeks before a significant increase in fatty infiltration occurred. The pre-Aureomycin biopsy (Fig. 5) disclosed a nodular type of architecture with marked portal scarring, lobular disarray, and evidence of considerable hepatic cellular and nuclear degenerative changes. Fatty metamorphosis was minimal at this time. Biopsy on the forty-fourth day, after fourteen days of Aureomycin, revealed the hepatic cells to be the site of very severe fatty changes with only a rare cell being spared (Fig. 6). On the fifty-eighth day, fourteen days after Fig. 6, and with Aureomycin and methionine during the interim, the liver showed the same amount of fat and a tendency toward coalescence of adjacent fatty droplets (Fig. 7). The final biopsy on

the sixty-eighth day, ten days after Aureomycin and methionine were discontinued, disclosed a marked decrease in the amount of fat with only single droplets being present in many lobules (Fig. 8). Periportal scarring and lobular disarray were still evident. In the other three patients no significant effect on the liver was noted during the administration of Aureomycin, but it should be emphasized that these five- to seven-day periods of Aureomycin administration were considerably shorter than that in J. D. It is also to be noted that the administration of Aureomycin to J. D. for the first week resulted in slight improvement in the histologic appearance of the liver as evidenced by complete disappearance of the fat.

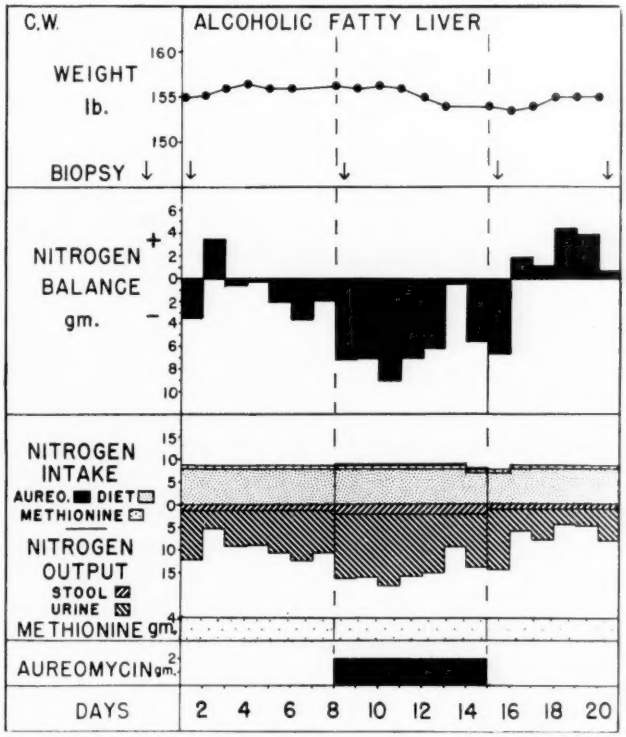


Fig. 4.—Negative nitrogen balance during Aureomycin therapy despite methionine.

Liver function tests in J. D. became slightly more abnormal during the administration of Aureomycin, but this change was probably not significant. In the other three patients these tests were either unaltered or continued to improve during Aureomycin ingestion. Patient W. H. with convalescent hepatitis showed a steady decrease in serum bilirubin and thymol turbidity during the period of Aureomycin administration. The cephalin flocculation also improved slightly, as did the bromsulfalein retention.

*Eosinophil and Cortin Excretion Studies.*—Adrenal cortex function was studied by daily eosinophil counts and repeated urine cortin determinations on

TABLE III. EOSINOPHIL COUNTS AND URINE CORTIN EXCRETION DURING AUREOMYCIN, PATIENT C. W.

| DAY | EOS./ CU. MM. | CORTINS MG. | DAY | EOS./ CU. MM. | CORTINS MG. |
|---|---|---|---|---|---|
| 1 | 122 | - - | 11 | 132 | - - |
| 2 | 99 | - - | 12 | 220 | - - |
| 3 | 299 | - - | 13 | 199 | 0.6 |
| 4 | 440 | - - | 14 | - - | 0.6 |
| 5 | 110 | 1.16 | 15 | 266 | - - |
| 6 | 155 | - - | 16 | 177 | - - |
| 7 | - - | - - | 17 | 388 | - - |
| 8* | 144 | 0.5 | 18 | 133 | 0.76 |
| 9 | 133 | - - | 19 | 122 | 0.43 |
| 10 | 277 | - - | 20 | - - | - - |

Normal cortin excretion 0.23 to 0.75 mg. daily.
*Aureomycin 2 Gm. daily from eighth day to fourteenth day, inclusive.

patient C. W. No significant alterations in either of these determinations occurred during the Aureomycin period (Table III).

## DISCUSSION

The results obtained indicate that increased nitrogen excretion occurs consistently during the administration of Aureomycin, but such nitrogen loss is not necessarily accompanied by fatty infiltration of the liver. Whether negative nitrogen balance precedes fatty infiltration or is entirely independent of it cannot be determined from these studies, but the possibility exists that prolonged negative nitrogen balance or increased nitrogen catabolism may produce increased hepatic fat. Suggestive evidence is present for this possibility in the results obtained in patient J. D., but the relatively short-term studies on the remaining patients yield no evidence on this point. Some data are available, however, on the duration of Aureomycin administration necessary to produce fatty infiltration from the work of Sborov and Sutherland.[7] Using Aureomycin over periods of thirty to sixty days they found increased liver fat in only seven of sixteen patients, and some patients developed no hepatic change even after sixty days of antibiotic. These results suggest that there are individual variations in susceptibility of patients to fatty infiltration without definite relation to the duration of therapy. Lepper and associates[8] also have suggested that larger doses of Aureomycin increase the possibility of fatty infiltration. Although the studies reviewed[7] do not include data on nitrogen metabolism, it appears that if negative nitrogen balance was induced in all patients it did not correlate with fatty infiltration. In view of our results the weight gain observed in some patients by Sborov and Sutherland is difficult to explain unless deposition of fat or water retention far exceeded the loss of body protein. Marked increase in body fat has been found in animals as a result of Aureomycin therapy.[18]

Methionine deficiency as a cause of Aureomycin-induced protein catabolism appears to be unlikely in that no effect in reducing the nitrogen loss during Aureomycin administration was observed when methionine supplements were given to three patients. While the effect of methionine on the reversal of fatty infiltration of the liver induced by Aureomycin cannot be evaluated

adequately, the evidence from the first study (J. D.) suggests that methionine is unable to reverse fatty infiltration once it occurs during Aureomycin administration. The use of choline has not been studied in this regard, but an Aureomycin-induced choline deficiency would appear to be unlikely in view of the studies of de la Huerga and Popper who found that choline absorption was increased by the administration of Aureomycin.[19] This finding renders improbable the hypothesis that choline deficiency produces the fatty infiltration of the liver observed during Aureomycin therapy.

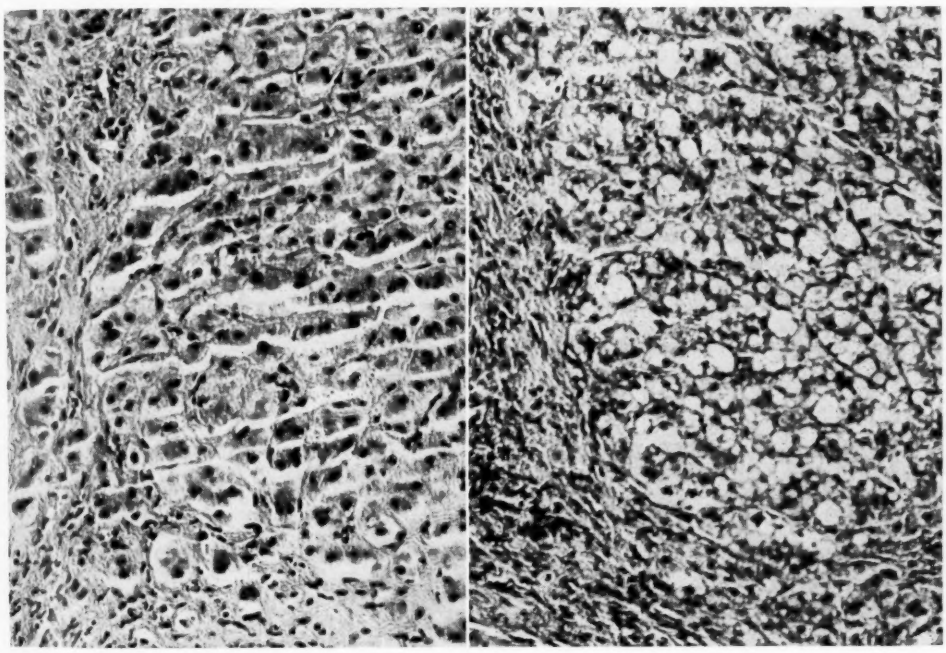

Fig. 5. Fig. 6.

Fig. 5.—Liver biopsy, J. D., twenty-seventh day of study, before Aureomycin administration. The portal scarring, lobular disarray and hepatic cell degenerative changes are marked and there is no evidence of fatty metamorphosis. (Hematoxylin and eosin stain ×265.)

Fig. 6.—J. D., biopsy forty-fourth day, after fourteen days of Aureomycin administration. Lobule showing evidence of severe fatty metamorphosis in nearly every hepatic cell. (Hematoxylin and eosin stain × 265.)

In connection with this action of Aureomycin, Baxter and Campbell[20] have observed that Aureomycin reduced the mortality, renal damage, and, to a lesser extent, the liver lipid content of rats receiving choline deficient diets. Their results are of particular interest in regard to liver lipids and suggest that either species differences, dosage, or diet variation may account for the protective effects they noted which are in contrast to the findings in human beings and in rats studied by other workers.[6-9]

No significant effect of Aureomycin on nitrogen or fat absorption in these patients was observed. The character of the stools was unchanged, and it is apparent that in the absence of diarrhea, the antibiotic does not increase stool nitrogen or fat loss. Although Merliss and Hoffman[5] have reported the devel-

opment of steatorrhea-like symptoms following antibiotic therapy, we have not observed this in our patients, and our analyses reveal no tendency for stool fat to be increased. These results, however, may not apply to all patients receiving Aureomycin.

The mechanism of the catabolic or anti-anabolic effect of Aureomycin cannot be delineated, but the similar increased nitrogen loss observed during ACTH and cortisone plus the reports of fatty infiltration of the liver in animals following administration of these hormones[21, 22] and in man following cortisone[23] led us to study adrenal cortical function during Aureomycin administration. The results of these studies in one patient suggest that there is no stimulation of the adrenal cortex which could account for the negative nitrogen balance.

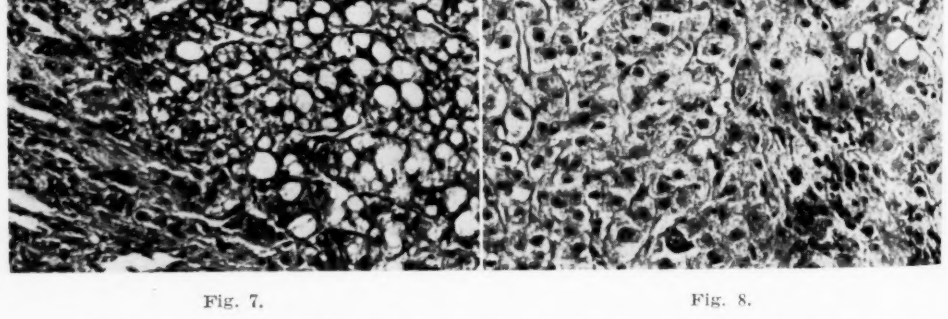

Fig. 7. Fig. 8.

Fig. 7.—J. D., biopsy fifty-eighth day, fourteen days after the biopsy shown in Fig. 6. Aureomycin and methionine treatment during interim. Lobules demonstrating severe fatty metamorphosis with a tendency for coalescense of adjacent droplets. (Hematoxylin and eosin stain ×265.)

Fig. 8.—J. D., biopsy sixty-eighth day, ten days after Aureomycin and methionine were discontinued. Nearly complete disappearance of the fatty changes although evidence of lobular disarray is still present. (Hematoxylin and eosin stain, ×265.)

The phenomena observed may be due either to a direct toxic effect on various enzyme systems or perhaps to interference in the utilization of certain vitamins such as riboflavin.[11] The studies of Gabuzda and his colleagues, however, do not eliminate the possibility that the increased excretion of riboflavin is but a reflection of the negative nitrogen balance. Excretion or retention of this vitamin has been demonstrated to parallel nitrogen anabolism or catabolism.[24]

Negative nitrogen balance induced by Aureomycin does not affect the removal of fat from the liver which normally occurs when a patient is offered an adequate diet. During brief periods of Aureomycin therapy in the two patients on whom balance studies were done, either no change or improvement in the fatty infiltration was observed. Even in patient C. W., the histologic appearance of the liver improved despite a relatively prolonged period of negative nitrogen balance due to nitrogen loss during the control period. This observation indirectly confirms the work of Eckhart and associates[25] who observed that negative nitrogen balance did not prevent clinical and liver function test improvement in patients with fatty cirrhosis.

The results of this investigation in human beings differ from the results observed in young fowls and animals in which Aureomycin has been reported to induce increased growth.[26] The differences observed may be due to smaller dosage used in the animal and fowl studies, to differences in the metabolic response of different species, or to differences in metabolic response between mature and young animals. Further studies with different dose schedules and continued investigation of the possible mechanisms of this protein catabolic or antianabolic effect of Aureomycin in human beings are indicated.

As a therapeutic agent for hepatitis and cirrhosis Aureomycin would appear to possess disadvantages as judged by these results. If fatty infiltration of the liver and increased nitrogen catabolism are used as criteria of deleterious effects upon liver disease, this agent should perhaps be used with caution. Final judgment, however, must be reserved in view of the relatively few patients studied and the lack of evidence of serious hepatic deterioration as judged by the function tests employed. In disease states in which protein anabolism is therapeutically desirable, however, the prolonged use of Aureomycin is probably undesirable.

## SUMMARY AND CONCLUSIONS

The effects of Aureomycin upon nitrogen metabolism, liver histology, and liver function tests were studied in three patients with alcoholic fatty cirrhosis and in one patient with convalescent hepatitis. In all patients urine nitrogen excretion was increased during antibiotic administration and in two subjects receiving constant diets negative nitrogen balance occurred. Nitrogen excretion was unaffected by the simultaneous administration of methionine.

Fatty infiltration of the liver was observed after two weeks of Aureomycin therapy in one patient, and was not reversed by the concurrent administration of methionine with Aureomycin for another two weeks. Reversion to pre-Aureomycin appearance of the liver was found ten days after Aureomycin was stopped. Liver histology was improved or unchanged in the three remaining patients who received Aureomycin for only five to seven days. Liver histology changes in one of these patients who received Aureomycin alone were not different from those in the two patients who received methionine and Aureomycin.

Stool nitrogen and fat were not significantly altered by Aureomycin in two patients so studied.

Liver function tests became slightly more abnormal in one patient in whom fatty infiltration was produced but were unchanged or improved in the other three patients. Blood eosinophil counts and urine "cortin" determinations in one patient were not significantly altered during Aureomycin.

The results obtained indicate that Aureomycin produces protein catabolism or anti-anabolism by mechanisms as yet unknown. Supplements of methionine do not reverse this effect on protein metabolism. No definite correlation between increased nitrogen excretion and fatty infiltration of the liver was observed in short (five to seven days) periods of Aureomycin administration.

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# THE EFFECTS OF LARGE DOSES OF CORTISONE ON TESTICULAR MORPHOLOGY AND URINARY GONADOTROPHIN, ESTROGEN, AND 17-KETOSTEROID EXCRETION

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ALTHOUGH elevated urinary gonadotrophin levels have been observed during cortisone and ACTH therapy,[1, 2] the mechanisms responsible for this increase are not clearly understood. The occurrence of amenorrhea, as well as diminished sexual potency and libido in patients receiving cortisone and ACTH[3, 4] suggests that *decreased* gonadal function may account for the increase in urinary gonadotrophin excretion. Other workers, however, have reported reappearance of menses following cortisone[5] and ACTH[6] therapy in young women having "secondary amenorrhea," suggesting a beneficial gonadal effect of cortical steroids. Obviously, more direct observations are necessary for determining the effects of cortical hormones on gonadal function.

Therefore, testicular biopsy specimens and twenty-four hour urine specimens for assay of gonadotrophins, estrogens and 17-ketosteroids were obtained before and during treatment with large amounts of cortisone in four adult men having rheumatoid arthritis. The objectives of this study were to determine the effects of cortisone on testicular function and thereby to obtain information concerning the mechanisms by which cortisone produces increased urinary gonadotrophin excretion.

The clinical features of each of the patients and the effects of cortisone therapy on the arthritis are described elsewhere in detail.[7] It is relevant to this discussion that no evidence of abnormal testicular function was obtained from history and physical examination in any of the patients. Each patient received intramuscularly every six hours an aqueous suspension of cortisone acetate, containing 25 mg. per cubic centimeter. The average daily dose was approximately 500 mg. The length of treatment before repeat studies were performed ranged from twenty-two to thirty-six days. Individual dosage schedules are listed in Table I.

## METHODS AND RESULTS

*Urinary gonadotrophins* were concentrated by ultrafiltration and assayed in immature female rats, as previously described.[8] The concentrate from a twelve-hour aliquot of urine was administered to each rat. Results are expressed

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in Table I as average ovarian weights. Gonadotrophin excretion increased in each of the three patients in whom assays were performed before and during therapy. In patient G. H. toxicity of extracts prepared from urine obtained before therapy prevented assay; gonadotrophin levels during therapy, however, were much higher than those present five months after stopping therapy. *During therapy* gonadotrophin excretion was greater than normal in each patient. *Before therapy* gonadotrophin excretion was not elevated, except in patient A. S., the only patient in whom pretreatment testicular biopsy revealed extensive damage.

*Urinary estrogens* were assayed in immature female rats, using uterine weight as the assay end point. The details of the method are described in a previous publication.[9] At least four rats were used in each assay. Alpha-estradiol benzoate was employed as the reference standard and results are expressed as the equivalent biological activity of this estrogen in micrograms per twenty-four hours. No marked alterations in estrogen excretion occurred during therapy. Urinary estrogen levels were within the range for normal men in patients J. M. and A. B. both before and during therapy. Patient A. S., in whom testicular biopsies revealed much less than normal numbers of Leydig cells, excreted less than normal amounts of estrogen before and during treatment. Patient G. H. excreted normal amounts of estrogen during therapy, but five months after therapy an unexplained high level was observed.

*Urinary 17-ketosteroids* were determined by the method of Drekter and associates.[10] Pretreatment values were within our range for normal men, except in patient A. S., in whom levels were definitely lower than normal. During cortisone therapy 17-ketosteroid excretion was 3 to 13 mg. greater than pretreatment levels.

*Testicular Morphology.—Before therapy* normal Leydig cells and only minor defects of seminiferous tubules were observed in three patients. Biopsy specimens obtained after twenty-two to thirty-six days of cortisone therapy revealed little change. Although less peritubular fibrosis was present during, than before, therapy in both patients A. B. and J. M., spermatogenesis appeared improved in patient A. B., whereas the repeat biopsy specimen of patient J. M. showed a tendency toward arrest of spermatogenesis. The seminiferous tubules of patient G. H. did not change remarkably. Leydig cell morphology during therapy was quite similar in each instance to that present before therapy. In patient A. S. severe defects of both seminiferous tubules and Leydig cells were observed before therapy and these defects were not remarkably altered by the administration of cortisone.

*Seminal fluid specimens* were collected by patients G. H. and J. M. before, during, and after therapy. Two specimens obtained during the week preceding therapy contained 151 and 76 million sperm per milliliter in patient G. H. and 164 and 125 million sperm per milliliter in patient J. M. These somewhat high numbers of sperm may have been related to absence of ejaculation for several weeks preceding collection of samples. Specimens collected at weekly intervals during treatment contained similar numbers of sperm in both patients, ranging from 25 to 58 million per milliliter. Specimens collected by patient G. H. five,

TABLE I. TESTICULAR MORPHOLOGY AND GONADOTROPHIN, ESTROGEN AND 17-KETOSTEROID EXCRETION BEFORE AND DURING CORTISONE THERAPY

| PATIENT | TIME STUDIES PERFORMED | TESTICULAR MORPHOLOGY | | URINARY HORMONE LEVELS | | |
|---|---|---|---|---|---|---|
| | | | | GONADOTROPHINS | ESTROGENS | 17-KETOSTEROIDS |
| | | LEYDIG CELLS | SEMINIFEROUS TUBULES | OVARIAN WEIGHT OF ASSAY RATS (MG.)* | MICROGRAMS PER 24 HOURS | MG. PER 24 HOURS |
| J. M. age 32 | Before therapy | Normal in number and appearance | Left: normal spermatogenesis with slight peritubular fibrosis and sloughing<br>Right: moderate peritubular fibrosis with some sloughing and disorganization of germ cells | 17.3 (4) | 1.3 | 12.2 |
| | After 22 days of Cortisone:<br>500 mg. q.d. for 19 days<br>200 mg. q.d. for 1 day<br>300 mg. q.d. for 1 day<br>500 mg. q.d. for 1 day | Normal in number and appearance | Left: decreased production of mature sperm, tendency to arrest at secondary spermatocyte stage, less peritubular fibrosis<br>Right: less peritubular fibrosis, less sloughing of germ cells | 80.7 (3) | 1.0 | 25.7 |
| A. S. age 50 | Before therapy | Much less than normal numbers; those present are vacuolated | Left: severe to complete sclerosis of most tubules, spermatogenesis poor<br>Right: moderate to severe fibrosis of all tubules, marked disorganization and sloughing of germ cells | 73.6 (5) | 0.3 | 5.8 |
| | After 35 days of Cortisone:<br>500 mg. q.d. for 35 days | Slightly more numerous, both large and small cells present | Left: less fibrosis<br>Right: fibrosis about same. In both testes sperm production slightly decreased; sloughing and disorganization about same. | 113.3 (6) | 0.4 | 14.6 |
| G. H. age 48 | Before therapy | Slightly less numerous than average; normal in appearance | Left: no biopsy specimen obtained<br>Right: majority of tubules show mild to moderate peritubular fibrosis, moderate hypoplasia and sloughing of germ cells; ⅓ of tubules severely or completely sclerosed | 9.4 (4)† | 2.5† | 15.8<br>16.4† |
| | After 36 days of Cortisone:<br>100 mg. q.d. for 6 days<br>300 mg. q.d. for 16 days<br>500 mg. q.d. for 14 days | Numbers slightly decreased, more vacuolated cells | Left: less fibrosis and hypoplasia and more sperm being produced than in right testis<br>Right: no change in fibrosis, germinal hypoplasia and sloughing still present | 60.4 (2) | 1.0 | 18.7 |

TABLE I. CONT'D

| PATIENT | TIME STUDIES PERFORMED | TESTICULAR MORPHOLOGY | | URINARY HORMONE LEVELS | | |
|---|---|---|---|---|---|---|
| | | | | GONADOTROPHINS | ESTROGENS | |
| | | LEYDIG CELLS | SEMINIFEROUS TUBULES | OVARIAN WEIGHT OF ASSAY RATS (MG.)* | MICROGRAMS PER 24 HOURS | 17-KETOSTEROIDS MG. PER 24 HOURS |
| A. B. age 42 | Before therapy | Normal in number and appearance | Left: spermatogenesis in progress, but moderate disorganization, sloughing and peritubular fibrosis<br>Right: majority of tubules show severe peritubular fibrosis | 11.0 (2) | 1.0 | 11.2 |
| | After 25 days of Cortisone:<br>500 mg. q.d. for 20 days<br>800 mg. q.d. for 5 days | Normal in number and appearance, relatively more small Leydig cells | Left: spermatogenesis improved with less peritubular fibrosis, less disorganization and less sloughing<br>Right: fewer tubules show severe peritubular fibrosis | 79.8 (2) | 0.6 | 17.8 |
| | | | Normals‡ | 10 to 40 mg. | 0.6 to 1.3 | 8.0 to 25.0 |

Figures in parentheses indicate number of rats per assay.
*Each rat received the concentrate of a twelve-hour aliquot of urine.
†Urine specimens collected five months after stopping therapy.
‡Data for normals are given in references [8] and [9].

nine, sixteen, and one hundred twenty-seven days after stopping therapy contained 34, 17, 18, and 21 million sperm per milliliter, respectively. The seminal fluid of patient J. M. collected four, seventeen, and one hundred seventeen days after stopping therapy contained 25, 102, and 25 million sperm per milliliter, respectively. No significant alterations were observed in sperm motility and morphology, or seminal fluid volume.

None of the patients noted any change in libido or number of erections.

## DISUSSION

The absence of regressive changes in testicular morphology suggests some explanation other than depressed gonadal function to explain the increased urinary gonadotrophin excretion observed during cortisone therapy. Smith has suggested increased renal clearance to account for the elevation in urinary gonadotrophins.[2] If this were the only mechanism, renal loss of gonadotrophins should result in decreased blood levels of hormone, and consequently regressive changes in testicular morphology might be expected. Since such changes were not observed, increased pituitary secretion of gonadotrophins, resulting in elevated blood levels of hormone, is postulated as a more plausible explanation for the increased excretion of gonadotrophins in patients receiving cortisone.

Although definite elevations in gonadotrophin excretion were demonstrated, the assays did not indicate whether follicle-stimulating hormone (FSH), interstitial cell-stimulating hormone (ICSH, also known as luteinizing hormone, LH), or both, were increased. Since human Leydig cells respond to stimulation with ICSH by producing increased amounts of estrogen,[9, 11] and since estrogen excretion did not increase in these men receiving cortisone, it is concluded that ICSH secretion was not appreciably altered. Thus, the elevation in urinary gonadotrophins is most likely a result of increased pituitary FSH secretion. The lack of seminiferous tubule and seminal fluid changes in these patients receiving cortisone does not preclude elevated blood levels of FSH, since it has not been demonstrated that adult human seminiferous tubules are capable of undergoing recognizable changes in response to *greater than normal* levels of FSH.

Although, to our knowledge, there is no information, other than our data, concerning the effect of cortisone on estrogen excretion in "normal" men or women, Nathanson and co-workers[12] found little change in photofluorometrically determined "estrogens" in men receiving ACTH. In women treated with ACTH, however, they observed a marked increase in estrogen excretion. Sprague, Albert and co-workers,[3] using a biological method of assay, have also observed increased estrogen excretion in women treated with ACTH. This apparent difference in response of the sexes to ACTH may be explained by the hypothesis that excess cortisone (or similar cortical steroids) causes increased pituitary secretion of FSH, but not ICSH. Although FSH, acting in conjunction with ICSH, stimulates ovarian estrogen secretion,[13, 14] in the male, ICSH, not FSH, stimulates Leydig cells[13, 14] resulting in increased estrogen production.[11] In accord with these concepts, we have observed that a pituitary gonadotrophin

preparation containing mainly FSH produces a marked increase in estrogen excretion when administered to women, but little change when administered to men.[15]

Another possible explanation for the increased gonadotrophin excretion observed during cortisone and ACTH therapy is that adrenal cortical steroids might produce increased urinary excretion of a nonhormonal "augmentor." This hypothesis, however, would not explain the increased estrogen excretion observed in women, but not in men, receiving ACTH. Furthermore, we have been unable to detect any augmenting substances in the urine of a man receiving cortisone (75 mg. orally per day) for the treatment of hypopituitarism. Extracts of his urine, which did not contain detectable amounts of gonadotrophin, failed to augment human gonadotrophin prepared from urine of a postmenopausal woman.

Only moderate increases in 17-ketosteroid excretion were encountered, even in the patient who was receiving 800 mg. of cortisone acetate daily when repeat studies were performed. The changes were similar to those found by other workers using much smaller doses,[3, 4] suggesting that the mechanism for conversion of cortisone to 17-ketosteroids is easily "saturated," thus limiting this pathway of metabolism of cortisone.

## SUMMARY AND CONCLUSIONS

Testicular function was studied in four adult men who received large doses of cortisone for the treatment of rheumatoid arthritis. Testicular biopsy specimens and urine for assay of gonadotrophins, estrogens, and 17-ketosteroids were obtained before and after twenty-two to thirty-six days of therapy, during which time each patient received a total of 10.5 to 17.5 Gm. of cortisone acetate. Although gonadotrophin excretion increased in each patient during therapy, no consistent changes occurred in testicular morphology or estrogen excretion. It is concluded that these observations are best explained by the hypothesis that cortisone causes an increased pituitary secretion of follicle-stimulating hormone (FSH), with relatively little change in interstitial cell-stimulating hormone (ICSH) secretion.

Increases in 17-ketosteroid excretion were similar to those found by other workers using smaller doses, suggesting that this pathway of metabolism of cortisone is easily "saturated."

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# BILIRUBIN AND PROTEIN IN THE CEREBROSPINAL FLUID OF JAUNDICED PATIENTS WITH SEVERE LIVER DISEASE WITH AND WITHOUT HEPATIC COMA

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IN 1918 van den Bergh[1] applied the Ehrlich-Pröscher diazo reaction for bilirubin to its detection in biological fluids. There has been disagreement regarding the nature of xanthochromic cerebrospinal fluid in jaundice, Froin's syndrome, meningitis, and following hemorrhage. Bilirubin has been demonstrated by chemical means in the cerebrospinal fluid in hepatic and obstructive jaundice,[2-5] Weil's disease, epidemic and tuberculous meningitis,[2] and the Froin syndrome.[6] Bilirubin occurs physiologically in the cerebrospinal fluid of premature infants and icterus neonatorum.[7, 8] The statement is frequently encountered[8-10] that because of the blood plasma-cerebrospinal fluid barrier, bilirubin occurs in the cerebrospinal fluid only in severe icterus of long standing, particularly in obstructive icterus. Also it has been stated[10] that the occurrence of xanthochromic cerebrospinal fluid in a patient with mild icterus is a point in favor of the diagnosis of Weil's disease. In view of a somewhat confused and conflicting literature, the present study was undertaken.

## MATERIAL AND METHOD

The control group consisted of 18 normal men. The serum bilirubin and the cerebrospinal fluid protein, cell count, and bilirubin content were determined. These same determinations were done on 10 consecutive patients with decompensated portal cirrhosis of the liver who had been jaundiced from one to four weeks. Eleven consecutive patients in hepatic coma (7 with portal cirrhosis and 4 with homologous serum hepatitis) were similarly investigated. These patients presented neurological manifestations which included mental confusion, listlessness or agitation, hallucinations, disorientation, somnolence, and profound coma. Neurological findings included hyporeflexia, absent response to painful stimuli, presence of sucking reflex, positive Babinski response, and fecal and urinary incontinence. It should be mentioned that the cerebrospinal fluid Wassermann reaction was negative in all subjects included in this study.

The serum and the cerebrospinal fluid bilirubin were determined according to the method of Malloy and Evelyn[11] as modified by Ducci and Watson,[12] and the cerebrospinal fluid protein by the method of Ling.[13]

## RESULTS

In the control group the mean blood serum bilirubin was 0.10 ± 0.03 mg. per cent in one minute with a total of 0.76 ± 0.30 mg. per cent (Table I). No

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bilirubin was found in the spinal fluid. The cerebrospinal fluid protein was found to have a mean value of 49.28 ± 9.27 mg. per cent. The leukocyte count ranged from 0 to 4 per cubic centimeter.

As shown in Tables I and II, bilirubin was present in the cerebrospinal fluid of 8 of the 10 jaundiced patients with decompensated portal cirrhosis, with a mean value for the group of 0.08 mg. per cent in one minute, and a total of 0.11 mg. per cent. The corresponding blood serum bilirubin was found to have a mean value at one minute of 8.06 ± 5.47 mg. per cent, with a total of 13.08 ± 7.88 mg. per cent (Table II). The cerebrospinal fluid protein had a mean value of 48.40 ± 13.79 mg. per cent which was not significantly different on comparison to the normal. The cell count was not increased (Tables I and II).

Also as seen in Tables I and III, bilirubin was present in the cerebrospinal fluid of 10 of 11 patients in hepatic coma with a mean value of 0.26 ± 0.08 mg. per cent at one minute and a total of 0.35 ± 0.14 mg. per cent. The correspond-

TABLE I. MEAN VALUES OF SERUM AND CEREBROSPINAL FLUID IN THE NORMAL AND IN LIVER DISEASE

| | | SERUM | | CEREBROSPINAL FLUID | | | |
|---|---|---|---|---|---|---|---|
| | | BILIRUBIN (MG. %) | | BILIRUBIN (MG. %) | | PROTEIN | W.B.C. |
| GROUP | NO. | 1′ | TOTAL | 1′ | TOTAL | (MG. %) | (NO.) |
| Normal | 18 | 0.10 | 0.76 | 0.0 | 0.0 | 49.3 | 0-4 |
| Cirrhosis | 10 | 8.1 | 13.1 | 0.08 | 0.11 | 48.4 | 0-2 |
| Hepatic coma | 11 | 11.1 | 16.2 | 0.26 | 0.35 | 98.9 | 0-4 |

TABLE II. SERUM AND CEREBROSPINAL FLUID VALUES IN DECOMPENSATED PORTAL CIRRHOSIS

| | SERUM | | CEREBROSPINAL FLUID | | | |
|---|---|---|---|---|---|---|
| | BILIRUBIN (MG. %) | | BILIRUBIN (MG. %) | | PROTEIN | W.B.C. |
| PATIENT | 1′ | TOTAL | 1′ | TOTAL | (MG. %) | (NO.) |
| R. H. | 15.0 | 22.0 | 0.1 | 0.1 | 58 | 1 |
| R. C. | 5.0 | 9.0 | 0.2 | 0.2 | 62 | 2 |
| R. C. | 15.0 | 21.5 | 0.0 | 0.1 | 54 | 0 |
| H. M. | 10.5 | 17.0 | 0.2 | 0.2 | 58 | 2 |
| A. C. | 13.5 | 21.5 | 0.0 | 0.0 | 31 | 0 |
| J. J. | 2.5 | 4.5 | 0.1 | 0.1 | 56 | 1 |
| J. K. | 2.2 | 4.7 | 0.0 | 0.0 | 40 | 0 |
| L. P. | 9.7 | 16.7 | 0.1 | 0.1 | 57 | 0 |
| A. H. | 6.8 | 13.0 | 0.1 | 0.2 | 20 | 2 |
| L. W. | 0.4 | 0.9 | 0.0 | 0.1 | 48 | 0 |
| x | 8.06 ± 5.47 | 13.08 ± 7.88 | 0.08 | 0.11 | 48.40 ± 13.79 | 0-2 |

ing blood serum had a mean bilirubin of 11.09 ± 7.11 mg. per cent at one minute, with a total of 16.20 ± 9.37 mg. per cent. The cerebrospinal fluid protein value was elevated in each of this group, with a mean value of 98.91 ± 37.50 mg. per cent (Tables I and III). Contrasted with the protein value of the control group this increase is highly significant ($p < 0.01$). The cell count was not increased.

## DISCUSSION

It has been appreciated in the past that the presence of bilirubin in the cerebrospinal fluid is abnormal. It would appear that in patients with severe

disease of the liver bilirubin appears in the cerebrospinal fluid more commonly than has been appreciated generally. It is also evident that the jaundice need not be intense nor of long duration. The bilirubin in the cerebrospinal fluid is almost quantitatively the prompt reacting variety which is probably due to a diffusion from the stained tissue into the cerebrospinal fluid. (A comparison of differences of the mean between the one minute and total reacting bilirubin was not significant.) There was no correlation between the amount of bilirubin in the cerebrospinal fluid and the degree of hyperbilirubinemia: i.e., patient A. C. showed no bilirubin in the cerebrospinal fluid when the serum value was 13.5 mg. per cent at one minute and a total of 21.5 mg. per cent.

Particularly noteworthy was the demonstration of a significant increase in the cerebrospinal fluid protein of the group in hepatic coma which has not previously been reported. The cause of this is not clear, although it may reflect damage to the central nervous system. The cerebrospinal fluid was abnormal in each patient in hepatic coma, the protein being elevated in all and bilirubin present with but one exception. Without awareness of this phenomenon it can be appreciated how confusing might be the finding of xanthochromic cerebrospinal fluid with elevated protein content in a patient presenting with the neurological manifestations of hepatic coma and whose liver disease has not been recognized.

TABLE III. SERUM AND CEREBROSPINAL FLUID VALUES IN HEPATIC COMA

| | SERUM | | CEREBROSPINAL FLUID | | | |
|---|---|---|---|---|---|---|
| | BILIRUBIN (MG. %) | | BILIRUBIN (MG. %) | | PROTEIN | W.B.C. |
| PATIENT | 1′ | TOTAL | 1′ | TOTAL | (MG. %) | (NO.) |
| H. P.* | 1.1 | 3.5 | 0.0 | 0.0 | 97 | 2 |
| E. I.* | 21.0 | 29.0 | 0.3 | 0.4 | 100 | 0 |
| D. S.* | 7.2 | 10.8 | 0.1 | 0.2 | 108 | 2 |
| B. S.† | 7.7 | 13.8 | 0.4 | 0.4 | 92 | 1 |
| C. A.† | 4.5 | 5.0 | 0.0 | 0.2 | 84 | 3 |
| C. L.† | 7.8 | 13.0 | 0.2 | 0.2 | 66 | 0 |
| C. H.* | 15.5 | 23.0 | 0.2 | 0.5 | 75 | 2 |
| J. H.† | 17.5 | 21.5 | 0.8 | 0.9 | 202 | 4 |
| A. H.* | 3.2 | 6.1 | 0.1 | 0.1 | 62 | 1 |
| H. K.* | 17.5 | 27.5 | 0.6 | 0.7 | 106 | 2 |
| C. J.* | 19.0 | 25.0 | 0.2 | 0.3 | 96 | 3 |
| $\bar{x}$ | 11.09 ± 7.11 | 16.20 ± 9.37 | 0.26 ± 0.08 | 0.35 ± 0.14 | 98.91 ± 37.50 | 0-4 |

*Cirrhosis, alcoholic.
†Homologous serum hepatitis.

## SUMMARY

Bilirubin was demonstrated in the cerebrospinal fluid of 8 of 10 jaundiced patients with decompensated portal cirrhosis. The cerebrospinal fluid protein was not increased in this group.

Bilirubin was demonstrated in the cerebrospinal fluid of 10 of 11 patients in hepatic coma (7 with portal cirrhosis, 4 with hepatitis). The cerebrospinal fluid protein was elevated in each instance, the difference of the mean value compared to the value of the control group being highly significant. This finding has not previously been reported. In no instance was the cell count altered from normal.

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# FILTER PAPER ELECTROPHORESIS WITH SPECIAL REFERENCE TO URINARY PROTEINS

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IN THE course of investigations relative to the application of zone electrophoresis in a filter paper support, a study of urinary proteins present in pathologic states was undertaken. This promised to be of interest in view of the findings already demonstrated by the method of electrophoresis in free solution.[1-7] The technical simplicity of the filter paper method augments the advantages that only very small quantities of material are required, and several analyses can be carried out simultaneously under identical conditions. The method has already proved useful in the characterization of sera[8, 9] and spinal fluid.[10] However, there have been evident certain difficulties in the physical setup as it has evolved thus far, and it became apparent early in the study of urinary proteins that some improvements in the basic technique were necessary.

This report deals with some of the technical problems and limitations encountered in filter paper electrophoresis and presents some of the findings regarding the great variation in the components of the urinary proteins.

## EXPERIMENTAL

1. *Methods.*—Random specimens of urine were collected from patients with a variety of diseases. Quantitative estimation of the urinary protein content was carried out using (a) the sulfosalicylic acid and methanol method of Bernard[11] and (b) the gravimetric method of Van Slyke.[12]

For purposes of filter paper electrophoresis only small quantities of material were necessary, each separation requiring 0.005 milliliter. Depending upon the amount of protein present 1 to 15 ml. of urine was concentrated to 0.2 to 0.3 ml. by dialysis in 25 per cent aqueous polyvinyl pyrrolidone (suggested by the work of Schneider and Wallenius with dextran[10]). Dialysis with barbital buffer was effective in resolving precipitates which appeared as the urines became increasingly concentrated.

The method of zone electrophoresis was essentially that described previously.[8] One strip of Whatman No. 3 MM filter paper was cut to fit between two glass plates, 11 by 23 by 0.5 cm., and to extend about 4 cm. beyond each end. After dampening the paper with barbital buffer pH 8.6, Γ/2 0.1, and blotting, 0.005 ml. of the material to be analyzed was applied with a specially calibrated micropipette* to pencil spots previously marked across the center of the paper. The spots were at least 1.5 cm. apart. After enclosing the paper between the siliconed surfaces of the glass plates, the sides were sealed with silicone grease, bulldog clamps placed on each side to press the plates together, and the free ends of the paper immersed in the tanks of electrolyte solution. Application of a direct current across the field effected good separation of the protein components in ten to twelve hours, depending upon the potential applied which was regulated by a potentiometer.

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A normal serum, to which a few crystals of bromphenol blue were added to label the albumin, served as a guide during the separation and as a normal control. The albumin was arbitrarily permitted to migrate 7.5 cm. from the origin. At the completion of the separation the paper was immersed for two minutes in a solution of 1 per cent bromphenol blue in 95 per cent alcohol saturated with mercuric chloride. It was then washed repeatedly with 1/1,000 acetic acid solution and dried at room temperature on a nonabsorptive surface.

All patterns were run in duplicate. One pattern was sectioned into 0.5 cm. segment and the dye quantitatively eluted by 1.5 ml. of 0.01N sodium hydroxide and the color read after one hour in a Coleman, Jr., colorimeter at 575 λ.

2. *Details Requiring Special Consideration.*—Under the conditions of the experiments, repeated tests revealed that too great a current intensity resulted in lack of uniform separation and some smearing of the components of the patterns. A relatively low current produced well-defined patterns. For example, with a potential of 12 to 15 volts per centimeter developing a current of 12 to 15 milliamperes for five to six hours, the pattern spots were not as well circumscribed as with a potential of 4 to 5 volts per centimeter and a current of 5 to 6 milliamperes operating for ten to twelve hours. These factors varied from one experiment to another depending upon the over-all resistance of the unit, which was determined by the size and dampness of the paper strip. However, the optimum field strength was readily ascertained after a few trials.

The moisture of the paper also played an important role in the eventual clarity of the pattern. If the paper was too moist with buffer when the protein spots were applied and during the separation, smearing occurred. It was found that after briefly encasing the dampened filter paper between sheets of absorbent blotter, a medium degree of moisture remained. Under these conditions 0.005 ml. of serum diffused to produce a spot 7 to 8 mm. in diameter.

Intrinsically associated with the moisture of the paper was the problem of uniform pressure between the glass plates. The method of paper electrophoresis used successfully by Durrum,[9, 13] employing a system with the paper suspended in an atmosphere saturated with buffer, avoids the pressure problem. However, this method also has some disadvantages, particularly relating to moisture regulation and control of evaporation and makes difficult the assessment of relative mobilities. In the present work a degree of pressure sufficient to maintain the paper in a moderately moist state was developed by four two-inch bulldog clamps using the size of plates described above. Occasionally irregular distribution of pressure resulted in local variation of buffer content in the paper, and consequently irregular patterns. A pressure of 1 lb. per square inch was found most suitable.

TABLE I. DIAGNOSIS AND URINARY PROTEIN VALUES OF THE CASES PRESENTED IN FIGS. 3, 4, 5, 6, 7, AND 8

| CASE | DIAGNOSIS | URINARY PROTEIN | |
|---|---|---|---|
| | | SULFASALICYLIC ACID PPT. | GRAVIMETRIC GM./LITER |
| (A) | Multiple myeloma | 2+ | 5.1 |
| (B) | Multiple myeloma | 4+ | 25.7 |
| (C) | Multiple myeloma | 2+ | 6.2 |
| (D) | Hepatitis | tr | .11 |
| (E) | Cirrhosis of the liver | 2+ | 6.5 |
| (F) | Nephrosis | 2+ | 7.9 |
| (G) | Nephrosis | 2+ | 7.7 |
| (H) | Nephrosis | 2+ | 7.4 |
| (I) | Mental retardation | 1+ | 1.1 |
| (J) | Nephrosis | 3+ | 11.6 |
| (K) | Nephrosis | 2+ | 2.5 |
| (L) | Nephrosis | 4+ | 44.4 |
| (M) | Nephrosis | 4+ | 24.0 |
| (N) | Nephrosis | 4+ | 24.0 |
| (O) | Nephrosis | 4+ | 24.8 |

In regard to the reproducibility of the staining method, two minutes' exposure of the patterns to a fresh solution of bromphenol blue sufficed to produce maximal color. Immersion of the paper for three minutes or longer achieved no greater intensity of color. Furthermore, the components of normal serum patterns produced simultaneously on the same sheet of paper showed very similar staining characteristics. When measured quantitatively by dye elution, the standard deviation was 0.01 O.D. unit.

In certain of the electrophoretic separations the dye-labeled albumin component flattened as it approached the anodal end of the paper. This resulted from the electro-osmotic flow of concentrated buffer from the exposed section of paper which was subject to evaporation. The anomaly was prevented by covering the exposed area with domestic waxed paper.

Adsorption represented the most serious limitation in the use of paper for zone electrophoresis. Slight trailing of the albumin component was a consistent finding. Radioautographs of tracer-labeled albumin illustrated the trailing in the path of migration. The adsorption of $\gamma$ – globulin was more pronounced than albumin. In recent studies[14] this anomaly was also shown to interfere with the characterization of serum lipoproteins and lysozyme. The adsorption is chiefly of an irreversible type that is not dependent on protein concentration. As a result it becomes most apparent with dilute protein solutions where a considerable portion of the protein is consumed in the path of migration. This

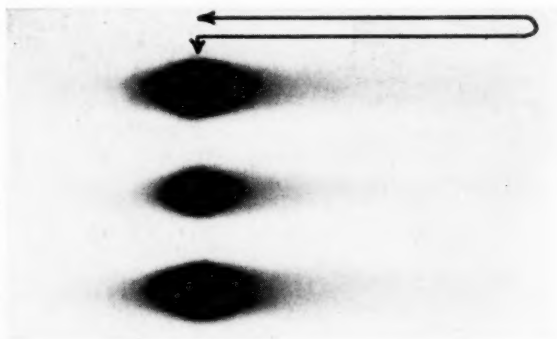


Fig. 1.—Illustration of the adsorption of protein by filter paper in the path of migration when (*A*) normal human serum, (*B*) 5 per cent human albumin, (*C*) serum from a patient with cirrhosis of the liver are returned to the origin by reversal of the current following electrophoretic separation. The trailing was more apparent before reproduction.

effect is somewhat less apparent in serum where the major protein components are at a high concentration. A number of methods can be used to determine the degree of trailing for a particular protein system. Two-dimensional electrophoresis was shown previously[8] to demonstrate the adsorption of serum proteins not apparent from one-dimensional experiment. Reversal of the current after separation of the proteins, permitting them to return to the origin, also represented a good method to demonstrate this phenomenon (Fig. 1). The adsorption could be determined most readily by permitting the protein to be carried through the paper by liquid flow without an electric current. Efforts to overcome this difficulty did not meet with success. Preliminary treatment with 6 per cent calcium disodium versenate, 1 per cent 8-hydroquinoline, 2N acetic acid, 1N calcium chloride, 1N magnesium sulfate, and 1N barium chloride did not produce any decrease in the adsorptive capacity of the paper. Munktell, Whatman No. 1 and No. 2 papers showed approximately the same absorptive qualities as Whatman No. 3 paper.

3. *Broad Paper Electrophoresis.*—It became apparent early in the study of urinary proteins that it would be desirable to separate a number of specimens on the same sheet of paper so that specimens of serum and urine could be compared directly under similar conditions. For this purpose a broad strip of filter paper, on which 14 patterns could be produced, was encased between thick glass plates measuring 23 by 25 by 2.5 centimeters. Preliminary experiments using relatively thin broad glass plates were unsuccessful because

the bending of the plates under pressure resulted in irregular migration. Thick glass plates insured even distribution of pressure to all parts of the paper. Nevertheless, slight irregularities of the patterns occurred in some separations which may have been caused by slight differences in the density of the paper in different areas. When the materials to be examined were applied identically to two sheets of filter paper, and one superimposed exactly upon the other, these small differences were minimized.

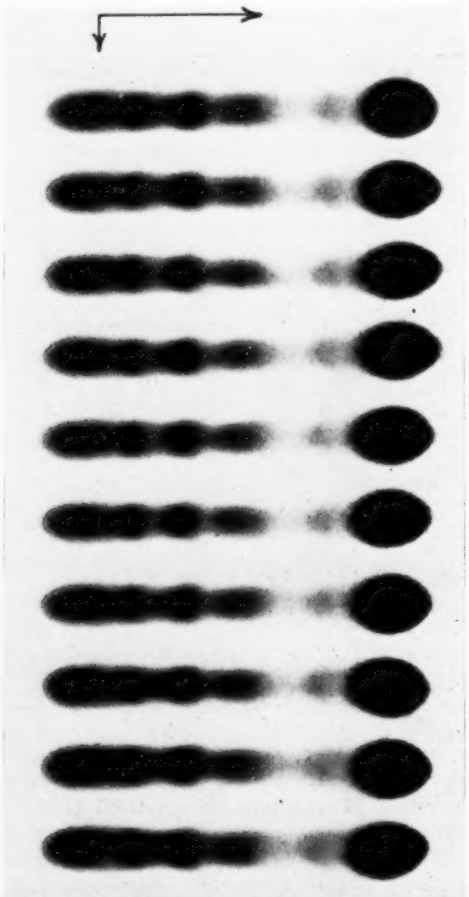

Fig. 2.—Ten electrophoretic patterns of a normal human plasma produced simultaneously on a broad paper strip. The spots from right to left are albumin, $\alpha_1$ – $\alpha_2$ – $\beta$–globulin, fibrinogen, and $\gamma$–globulin. The arrows indicate the origin and direction of migration.

Fig. 2 illustrates ten electrophoretic patterns of one normal human plasma produced simultaneously on a broad strip of filter paper. The classical components are readily recognized from the anodal right side as albumin, $\alpha_1$–, $\alpha_2$– and $\beta$-globulin, fibrinogen, and $\gamma$–globulin. Successful patterns were obtained when the plates were held firmly together by one C-clamp, the pressure being applied at the center of the plates.

Equilibration of the paper during assembly of the unit proved important in the production of successful patterns. This was carried out after dampening but before applying the aliquots of material to the paper and also after encasing the paper between the plates and immersing the ends in electrolyte solution. Slight flattening of the dye-labeled tracer

spot was produced before the current was applied. With attention to such details, patterns were closely reproducible, the distance of migration of the various components having a mean deviation of 1 mm. in a pattern of 120 mm. length.

Separations on the broad sheets of paper carried out at 20° C. with a current of 15 Ma. and potential of 3 to 4 volts per centimeter usually resulted in good patterns. Greater current intensity or higher environmental temperatures were accompanied by apparent denaturation and trailing of the fibrinogen component when plasmas were being separated. The patterns shown in Fig. 2 were carried out at 20° C. and have a clear-cut fibrinogen spot.

Fig. 3.—Normal serum and nine urines separated simultaneously on the same sheet of filter paper (*A* to *C*, multiple myeloma; *D* and *E*, liver disease; *F* to *H*, nephrosis; *I*, proteinuria of unknown origin). The arrows indicate the origin and direction of migration.

*Studies With Urinary Proteins.*—In the analysis of urinary proteins by filter paper electrophoresis, it was necessary to concentrate the urines to a variable degree, depending upon the protein content of the urine. This permitted the demonstration of all protein components which were present in any appreciable concentration. Table I presents the protein concentrations of the urine of some of the patients studied. Fig. 3 illustrates the urine patterns obtained from nine patients with various types of proteinuria. The marked variation in the type of proteins which may be present in the urine from different patients with the same disease as well as from one disease to another is apparent.

Four Bence-Jones urinary proteins were investigated. The individuality of these proteins is seen in the patterns of Figs. 3 (*A*, *B*, and *C*) which compare the findings of three cases of multiple myeloma. Two cases are shown in more detail in Figs. 4 and 5, which contrast the paper pattern and dye elution curve of the urinary and serum proteins.

Examination of the urines from ten patients with nephrosis brought out striking differences in the relative amounts of the protein components present in the urine compared to the corresponding serum. Three patterns of urinary proteins are shown in Fig. 3

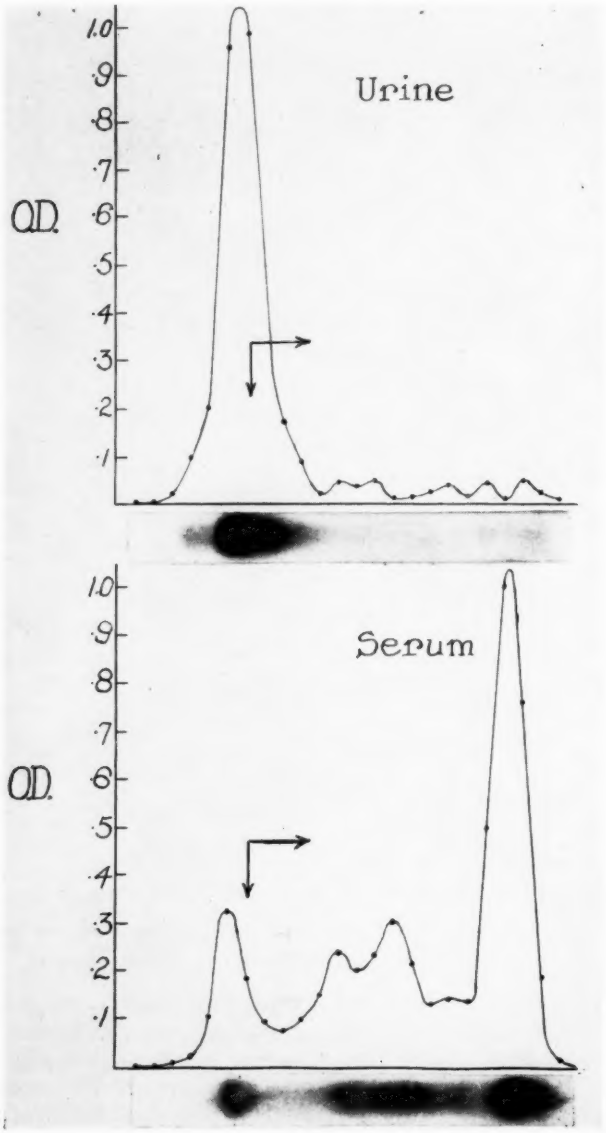


Fig. 4.—Comparison between the urine and serum proteins separated simultaneously from case (*B*) with multiple myeloma. The dye elution curves are illustrated along with duplicates of the paper patterns.

(*F*, *G*, and *H*). Fig. 6 illustrates the corresponding serum and urine patterns of five patients with nephrosis. It was apparent that each urine pattern was different, and that there was considerable variation in the relative amounts of urinary protein components from one case to another. A consistent finding was the high concentration of urinary albumin compared to the globulin components. As shown in Fig. 6, this occurred with or without any well-defined change in the serum protein pattern.

Of further interest was the absence in the urine of a spot corresponding to the large lipoprotein component seen in the sera of the more advanced cases of nephrosis. The lipoprotein was identified by lipid analyses on the isolated fractions as described previously,[15]

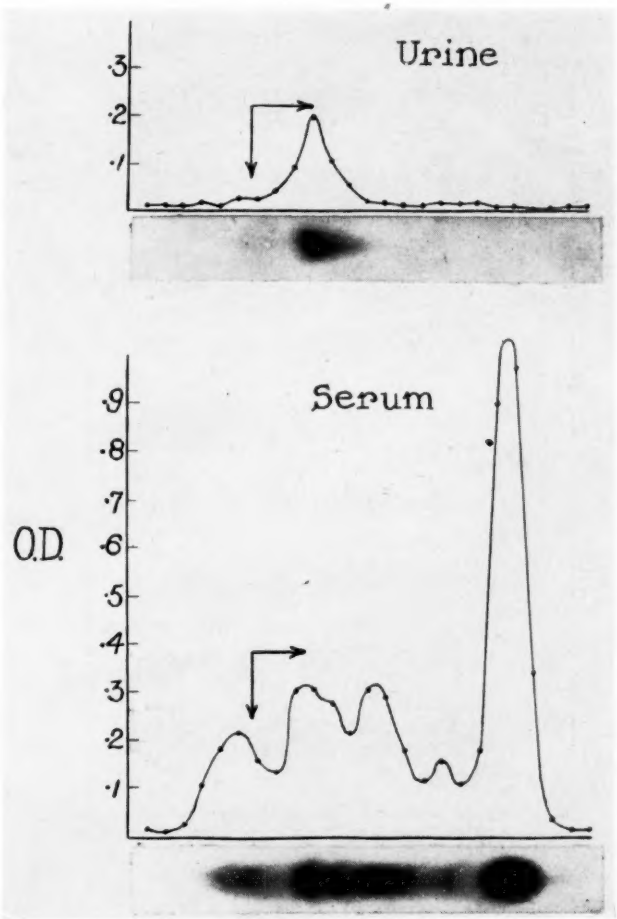


Fig. 5.—Corresponding urine and serum protein patterns separated simultaneously from case (*C*) with multiple myeloma. In this case the Bence-Jones protein has the mobility of a $\beta$-globulin.

and is visible as a densely staining elongated spot in the $\alpha_2 - \beta$ region of the serum patterns in Fig. 6. It is particularly marked in sera (*M*) and (*N*). Although the urine from these patients did not contain such lipoprotein spots, a sharply delineated $\beta$-globulin band was seen in every case. Fig. 7 contrasts the color patterns and dye elution curves of a corresponding serum and urine from a patient with nephrosis; the urinary $\beta$-globulin component

has a somewhat slower mobility than the large serum lipoprotein peak. In the urine from patients with nephrosis, the $\beta$-component appeared in every specimen containing albumin. Because of overlapping by the lipoprotein spot the serum counterpart of the urinary $\beta$-globulin band was not readily distinguished. However, it appeared to be relatively lower in the serum than the urine. Other protein components, particularly $\alpha$-globulins, were noted in certain urines without obvious counterparts in the corresponding sera (Fig. 6, *L* and *N*).

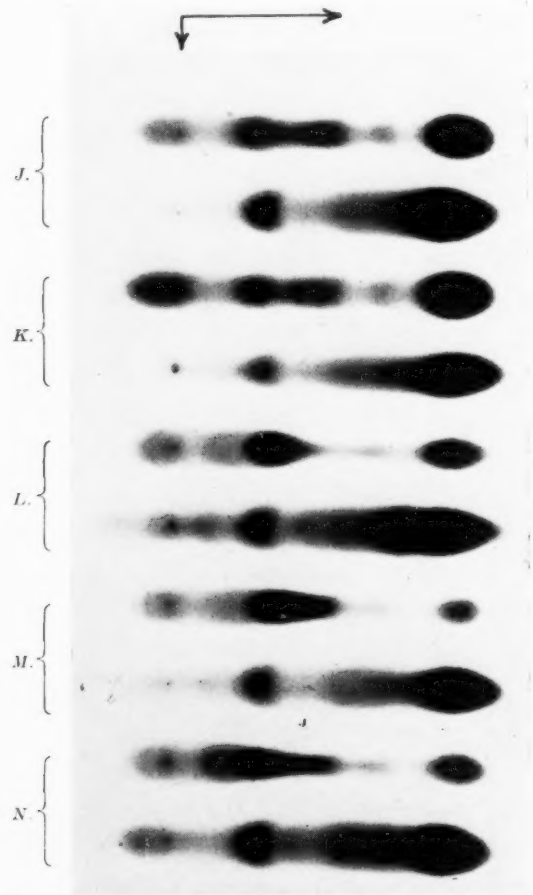


Fig. 6.—Serum (upper) and corresponding urine (lower) patterns from five cases of nephrosis separated simultaneously on one sheet of filter paper.

The urine obtained from two patients who had viral hepatitis exhibiting 1+ proteinuria was examined. In each case a well-defined albumin moiety was observed. However, other proteins of globulin mobilities were also present in variable quantity. The pattern of one case is shown in Fig. 3, *D*. Although this urine required fiftyfold concentration, three well-defined components are exhibited. Of these the slowly migrating component probably corresponds to the $\beta$-globulin component present in the urines from patients with nephrosis.

Urine from three patients with cirrhosis of the liver was studied. In only one patient who had persistent proteinuria were clear-cut patterns obtained. This individual's condition was remarkable because of a great elevation of the serum $\gamma$-globulin component, in the range of 6 Gm. per cent. Large quantities of protein were excreted in the urine the

characteristics of which are seen in Fig. 3, *E* and compared to the serum in Fig. 8. Although the γ-globulin was higher than in any other urine examined, the albumin/globulin ratio of the urine was still considerably higher than that of the serum.

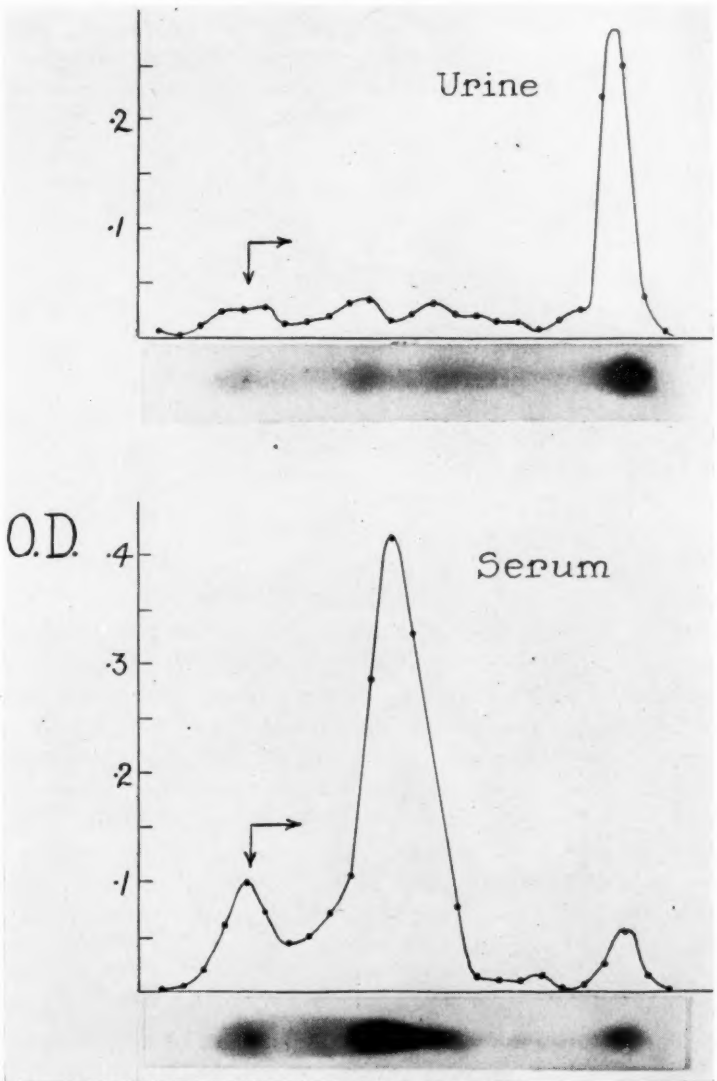


Fig. 7.—Corresponding urine and serum protein patterns separated simultaneously from case (D) with nephrosis. The tremendous lipoprotein component in the serum is not reflected in the urine pattern.

A random specimen of urine from an 8-year-old mentally retarded child with no history of renal disease was examined. Routine urinalysis revealed 1+ proteinuria. The electrophoretic pattern appears in Fig. 3, *I*. Again the preponderance of the albumin moiety was apparent. Furthermore, resemblance to the patterns of urine from nephrosis included the presence of the β-globulin component.

Attempts to characterize on paper the urine from three healthy subjects met with difficulty. In each case, 2 to 4 liters of urine were dialyzed against water and concentrated by vacuum distillation. Despite prolonged dialysis with water, saline, or barbital buffer, the urine concentrates remained viscous and brown in color, varying from one urine to another. Paper electrophoresis of these concentrates produced a smeared pattern which stained rather

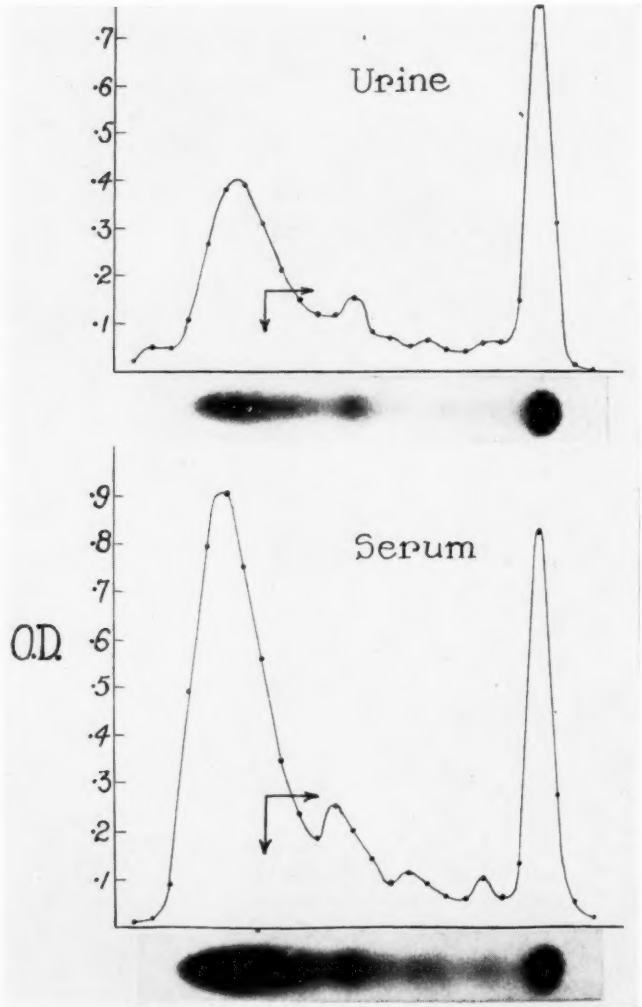


Fig. 8.—Corresponding urine and serum proteins separated simultaneously from case (*E*) with cirrhosis of the liver. The high $\gamma$-globulin in the serum was reflected in a high urinary $\gamma$-globulin.

poorly. However, components were apparent which corresponded to albumin and $\gamma$-globulin. There was also an ill-defined area in the $\beta$-globulin region. Considerable material remained adsorbed to the site at which the urine was applied to the paper. This sticky material was partially eliminated by preliminary precipitation of the mucoprotein from the urine by the method of Tamm.[16] However, this procedure did not produce any improvement in the appearance of the paper patterns.

## DISCUSSION

In the present study no attempt was made to perfect the quantitative measurements of the spots on the filter paper as described by other workers.[17, 18] Simple segmentation of the paper with elution of dye was utilized to produce the patterns. It appears likely that quantitative procedures applied to filter paper electrophoresis may be subject to considerable error because of technical variations and adsorption of the proteins in the path of migration. The adsorption is best observed by permitting serum to be carried by buffer flow without an electric current, and this should be done with all protein solutions before drawing conclusions regarding the electrophoretic separation. With some proteins the adsorption consumes so much material that no main spot is obtained except at high protein concentration. The principal effort in the present study was directed toward improving the basic separation of the serum and urine components by minor alterations in technique. The importance of employing well-blotted paper from which all visible moisture has been removed and the importance of a high evenly distributed pressure on the glass plates became apparent. No success was achieved in eliminating the adsorption of protein, and this represented the chief limitation of the method.

Adsorption of the proteins as well as other factors precluded the use of filter paper electrophoresis for the characterization of the normal urinary proteins. However, when as little as a trace of protein was observed by the sulfosalicylic acid test, such as is found in many disease states without obvious renal involvement, satisfactory patterns were obtained which could be interpreted.

In the disease, multiple myeloma, characterization of the Bence-Jones urinary protein on filter paper represented a simple confirmatory procedure which was of diagnostic value. The pattern of a single dominant protein peak in the urine other than albumin was unique and was not observed in other conditions. The presence of complicating renal disease in such patients could be detected readily by the appearance of other components.

Of special interest were the findings in one patient who had cirrhosis and markedly elevated $\gamma$-globulin levels in the serum. The urinary $\gamma$-globulin was greatly elevated compared to the values found in other cases of proteinuria. However, the $\gamma$-globulin level was higher in the serum relative to albumin than in the urine. This case demonstrated the relationship between the urinary protein components and the molecular size of the protein molecules. The small albumin moiety (molecular weight approximately 60,000[19]) passed the renal barriers more readily than the $\gamma$-gloublin moiety (molecular weight approximately 150,000[20]).

The urinary proteins of patients with nephrosis did not show all the major components of the corresponding serum, and a great divergence was seen. Only the albumin moiety was present in great quantity. In contrast, the tremendously increased amounts of lipoprotein found in the serum did not appear in the urine, apparently because of the high molecular weight (1,200,000[21]). Of particular interest was the appearance of a distinct $\beta$-globulin spot in the urine of patients with nephrosis, sometimes without a visible counterpart in the

serum. This was also observed in other conditions and suggests that a low molecular weight $\beta$-globulin is present in the serum which is readily cleared by the kidneys. It is possible that this urinary $\beta$-globulin is the serum iron-binding $\beta_1$-globulin (molecular weight approximately 90,000[22]) which has been shown by Lawson and Shulman[23] to be excreted in large amounts in the urine in patients with the nephrotic syndrome.

Chinard and co-workers,[24] in reviewing the electrophoretic data of other authors, noted the considerable variation in urinary proteins in patients with nephrosis. They concluded that in this disease the glomerular wall was permeable to plasma proteins in approximately inverse proportion to the size of the molecules. The patterns of urinary protein excretion in different diseases shown in the present study lend support to that theory.

Certain of the urinary proteins could not be classified, and their origin remains obscure. This was particularly true of those components without obvious counterparts in the serum. The possibility remains that these represent low molecular weight proteins produced under the stimulus of protein depletion that are cleared rapidly by the kidney and never reach measurable levels in the serum.

## SUMMARY

1. The use of broad strips of filter paper on which multiple electrophoretic separations could be carried out has been described. Specimens of serum and urine from the same patients could thus be separated simultaneously under identical conditions.

2. The limitations of filter paper electrophoresis, particularly those due to adsorption of protein, were discussed.

3. The method proved useful in the differential diagnosis of proteinuria, especially in cases of multiple myeloma with Bence-Jones urinary protein.

4. The dependence of the relative concentrations of urinary proteins on their molecular size was apparent in various conditions associated with proteinuria. The high molecular weight serum lipoproteins were not detectable in the urine.

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# PLASMA LEVELS AND THE RELEASE OF A LYSOZYME-LIKE ENZYME FROM TUBERCULIN-EXPOSED LEUKOCYTES OF TUBERCULOUS AND NONTUBERCULOUS HUMAN BEINGS

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THE question of a direct and specific cytotoxic effect of tuberculin on sensitized cells has been pursued by various investigators.[1-10] Studies concerning the in vitro response of tissue cultures of cells from tuberculin-sensitive guinea pigs or rabbits to added antigen have indicated an inhibitory effect in some instances[1-5] and no inhibition in others.[6, 7] Miller, Favour, and associates[8] used a different technique and demonstrated a cytolytic factor in tuberculous plasma which was effective on human leukocytes in vitro in the presence of tuberculin. Others[9, 10] have found disturbed phagocytic function of leukocytes from delayed-type sensitized guinea pigs on in vitro exposure of these cells to the specific antigen.

In the present report, nonspecific evidence of damage to leukocytes from tuberculous and nontuberculous human beings is observed on in vitro exposure of the cells to tuberculin, but no specific effect correlating with the tuberculous state of the subject is detected. The method used for the demonstration of cell injury[11] is based on the fact that certain cellular contents are released into the surrounding medium when cells are damaged. The substance selected for measurement is an enzyme with the properties of lysozyme.

During control studies, it was noted also that there is a significantly higher level of absolute lysozyme-like activity in tuberculous plasma than in nontuberculous plasma. The nontuberculous patients as a whole were not as ill as the tuberculous patients, although patients with terminal illness were excluded from both groups.

## MATERIALS AND METHODS

*Subjects.*—Human subjects were used as a source of leukocytes. Tuberculous subjects were in various stages of active tuberculosis, for the most part with tubercle bacilli demonstrable in the sputum, and in most instances without recent chemotherapy or antibiotic therapy. Control subjects were patients without evidence of tuberculous infection. They included patients with neurotic complaints, with infections other than tuberculosis, and with other diseases excluding primary metabolic or hematologic disorders.

The tuberculous patients used for the majority of the plasma determinations (but not for the leukocyte studies) were individuals with far-advanced tuberculosis, clinically fairly

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well controlled on streptomycin and PAS therapy but with tubercle bacilli demonstrable in the sputum.

*Leukocyte Suspension.*—Sedimented leukocytes prepared by a method described previously[11] were resuspended in physiologic saline plus 10 per cent autologous plasma to a final count of 5,500 leukocytes per cubic millimeter.

*Leukocyte Lysate.*—Sedimented leukocytes were resuspended in distilled water to a concentration equivalent to 5,500 leukocytes per cubic millimeter. This was diluted 1:10 in distilled water for use.

*Plasma.*—Cell-free plasma was diluted 1:10 in physiologic saline for use.

*Tuberculin.*—Tuberculin, Purified Protein Derivative* was diluted 0.1 mg. per milliliter of physiologic saline for use. Final dilution (see below) was 0.015 mg. per milliliter.

*Staphylococcus Toxin*†.—Diluted 1:5 in physiologic saline for use.

*Lysozyme Substrate.*—Bacto Lysozyme Substrate* stock suspension was prepared and diluted for use by a method described previously.[11]

*Experimental Procedure.*—Five-tenths milliliter of tuberculin or staphylococcal toxin or saline was added to 2.8 ml. of leukocyte suspension. The mixtures were incubated in 25 ml. sterile siliconed flasks in a Warburg shaker at 37° C. for two hours before use. Leukocyte lysate and plasma were used in the dilutions indicated previously without prior incubation. In each instance, 1.0 ml. of the test material was added to 1.5 ml. of lysozyme substrate, and readings were obtained at 0 time and at twenty minutes at 540 mμ in a Coleman Junior spectrophotometer, as previously described,[11] 100 per cent transmission being set by use of a blank tube containing 1.0 ml. of the test material and 1.5 ml. of buffer. All tests were run in duplicate. Per cent lysis of the substrate was calculated by the formula 100 minus (the optical density at twenty minutes divided by the optical density at 0, times 100). Where comparison of uninjured and injured leukocytes was desired, a correction for the rate of the enzymatic reaction in the control suspension was necessary, since differences noted in a fast-moving system cannot be compared directly with those observed in a slow-moving system. For this purpose, an index was used (see Table II), the derivation of which is described elsewhere.[11]

## RESULTS

The design and results of experiments are summarized in tabular form:

Table I indicates the per cent lysis of substrate observed in the presence of (A) leukocytes from two groups of human subjects, (B) leukocyte lysates from two groups of human subjects, and (C) 10 per cent plasma from two groups of human subjects. The individual composition of the two groups is not the same in the three categories tabulated. It will be noted that the per cent lysis

TABLE I. A COMPARISON OF THE LYSOZYME-LIKE ACTIVITY OF LEUKOCYTES, LEUKOCYTE LYSATES AND PLASMA FROM TUBERCULOUS AND NONTUBERCULOUS HUMAN BEINGS

| GROUP | PER CENT LYSIS OF SUBSTRATE IN THE PRESENCE OF: INTACT W.B.C. 5,500/CU. MM. | W.B.C. LYSATE DIL. 1:10 | PLASMA DIL. 1:10 |
|---|---|---|---|
| (A) Tuberculous patients | 36.3 ± 8.2 (23 patients) | 41.4 ± 7.8 (10 patients) | 15.1 ± 3.9 (30 patients) |
| (B) Nontuberculous patients | 31.4 ± 10.1 (52 patients) | 45.8 ± 8.6 (8 patients) | 10.3 ± 3.4 (28 patients) |
| Comparison of (A) and (B) | t = 2.031 p = (<0.05) | t = 1.131 p = (>0.05) | t = 4.937 p = (<0.01) |

*Obtained through the kindness of Dr. Florence Siebert of the Henry Phipps Institute, Philadelphia, Pa.

†Supplied through the courtesy of Dr. H. D. Piersma of Lederle Laboratories.

‡A dried preparation of *M. lysodeikticus*, supplied through the courtesy of Mr. H. W. Schoenlein of Difco Laboratories, Inc.

in the tuberculous group is somewhat greater than in the nontuberculous group, and that this difference is of possible significance ($p = <0.05$). This is not due to an increased enzyme content of tuberculous leukocytes, as indicated by the lack of significant difference in activity of leukocyte lysates in the two groups ($p = >0.05$). However, it may well be due to the greater activity of the plasma from the tuberculous group as compared to the nontuberculous group ($p = <0.01$). It will be recalled that 10 per cent autologous plasma was added to each leukocyte preparation.*

Table II indicates in the left hand columns the increase in per cent lysis of substrate as compared to the control when the leukocytes were previously exposed to tuberculin or to staphylococcal toxin. There is a greater increase in

TABLE II. THE EFFECT OF TUBERCULIN AND OF STAPHYLOCOCCAL TOXIN ON LEUKOCYTES FROM TUBERCULOUS AND NONTUBERCULOUS HUMAN BEINGS

| GROUP | PER CENT LYSIS OF SUBSTRATE IN EXCESS OF PER CENT LYSIS BY CONTROL, WITH ADDED | | INDEX CORRECTING FOR INITIAL SPEED OF REACTION (SEE TEXT, EXPERIMENT PROCEDURE) | |
|---|---|---|---|---|
| | PPD | STAPH. TOXIN | PPD | STAPH. TOXIN |
| (A) Tuberculous patients | 13.1 ± 6.2 (23 patients) | 19.5 ± 5.9 (11 patients) | 37.9 ± 16.2 (23 patients) | 60.5 ± 21.2 (11 patients) |
| (B) Nontuberculous patients | 9.7 ± 4.4 (26 patients) | 13.1 ± 7.5 (28 patients) | 36.0 ± 19.1 (26 patients) | 47.2 ± 31.9 (28 patients) |
| Comparison of (A) and (B) as | $t = 2.251$ $p = <0.05$ | $t = 2.513$ $p = <0.05$ | $t = 0.3732$ $p = >0.05$ | $t = 1.278$ $p = >0.05$ |

lysis over that observed in the control when tuberculous leukocytes are exposed either to tuberculin or to staphylococcal toxin, than on exposure of nontuberculous leukocytes to these agents. The differences between the tuberculous and nontuberculous groups in this respect are of possible significance ($p = <0.05$). However, when the data are corrected for the initial speed of the enzymatic reaction, as indicated in the right hand columns, the apparent differences between the two groups lose their significance ($p = >0.05$). The possible significance was an artefact resulting from the greater initial speed of reaction in the tuberculous group as compared to the nontuberculous group (see Table I).

## DISCUSSION

The present studies utilize the fact that cell injury is associated with the release of cell contents into the surrounding medium.[11] The substance selected for measurement has been an enzyme with the properties of lysozyme. Human leukocytes from tuberculous subjects have shown no greater evidence of damage after exposure for two hours to tuberculin than have leukocytes from nontuberculous patients. An apparent difference was found to be related to the presence of a greater quantity of the enzyme in tuberculous plasma than in nontuberculous plasma. We were unable also to demonstrate an increased effect of tuberculin on leukocytes in the few instances in which our studies were repeated on nontuberculous subjects thirty-six hours after the induction of a positive intracutaneous tuberculin test.

*Hemagglutination tests done on 26 of the 30 tuberculous plasma samples were positive in 19 instances. The titer showed no correlation with the lysozyme-like activity observed.

It is difficult to compare the present findings with those made by earlier investigators who used tissue culture techniques for studying the effect of tuberculin on sensitized cells, because the tissue culture observations recorded were usually made after longer periods of exposure of cells to tuberculin. Only Heilman and associates[4] specifically mention an inhibitory effect on migration observed as early as two hours after initial incubation. The experiments of Miller and Favour[8] are comparable with the present experiments as to exposure time after which a cytolytic effect was observed on sensitized cells in the presence of tuberculin, but the techniques used cannot be compared further.

A higher titer of lysozyme-like enzyme activity was found in tuberculous plasma than in nontuberculous plasma. While this may be related only to the fact that as a whole the nontuberculous patients were not as ill as the tuberculous patients, the finding is nonetheless of particular interest in view of the observations of Myrvik and Weiser.[12] In rabbits heavily immunized with heat-killed tubercle bacilli with Freund's adjuvants, they found an elevated titer of a tuberculostatic serum substance possessing lysozyme-like properties. The tuberculostatic substance was noted also in rabbits immunized with organisms other than tubercle bacilli. Our present state of knowledge concerning the factors governing the plasma level of the lysozyme-like enzyme reported here does not permit speculation at this time concerning the meaning of the observation.

## SUMMARY

1. By a method of detection of cell injury which depends on the release of intracellular substances from injured cells into the surrounding medium, the effect of tuberculin and of staphylococcal toxin on human leukocytes from tuberculous and nontuberculous subjects has been studied. The substance chosen for measurement is an enzyme with the properties of lysozyme.

2. No significant difference could be detected in the degree of cell injury resulting from two hours of in vitro exposure to tuberculin of tuberculous leukocytes as compared to nontuberculous leukocytes. The same observation was made using staphylococcal toxin.

3. Of high statistical significance was the greater degree of lysozyme-like enzyme activity in the plasma of tuberculous subjects as compared to nontuberculous subjects.

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# EFFECT OF ANTIHEMOPHILIC FACTOR ON ONE-STAGE CLOTTING TESTS

## A Presumptive Test for Hemophilia and a Simple One-Stage Antihemophilic Factor Assay Procedure

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BLOOD from patients with classical hemophilia has a prolonged clotting time, but when tissue thromboplastin is added as in the usual "prothrombin time" determination, hemophilic plasma clots as rapidly as does normal plasma.[1] The normal prothrombin time in hemophilia has apparently overshadowed other observations on the influence of thromboplastins on the clotting of hemophilic plasma. In 1926, Mills[2] showed that crude cephalin preparations which clotted normal plasma rapidly were relatively inactive when added to hemophilic plasma. In 1935, Eagle[3] found that platelet suspensions were similar to crude cephalin in this regard. In 1940, Dam and Venndt[4] showed that while tissue thromboplastins clotted hemophilic plasma rapidly, these same tissue extracts, if diluted, clotted hemophilic plasmas less rapidly than they did normal plasma.

In this study we have compared the effectiveness of several thromboplastins in accelerating the clotting of hemophilic and normal plasmas. On the basis of our findings it is suggested that thromboplastins may be classified as complete or partial. Complete thromboplastins clot normal and hemophilic plasmas equally fast, while partial thromboplastins clot hemophilic plasmas less rapidly than they do normal plasmas. Based on the differential reaction of hemophilic plasmas with complete and partial thromboplastins, we are proposing two new procedures: (1) a presumptive test for the diagnosis of hemophilia, and (2) a simple method for the assay of antihemophilic factor (AHF) in plasma. A preliminary report of this work was made recently.[5]

### MATERIALS AND METHODS

Both human and canine hemophiliacs were used in this study. Blood was obtained from 6 hemophilic human beings and 35 normal human beings by antecubital venepuncture, and from 20 hemophilic dogs[6] and five normal dogs by jugular venepuncture. The bloods were mixed immediately with anticoagulant, either 0.11M sodium citrate in the ratio of one part to 8 parts whole blood, or 0.10M sodium oxalate in the ratio of one part to 9 parts whole blood. Plasmas were obtained by centrifugation at about 3,000 *g* for twenty minutes. Oxatated saline was prepared by mixing one part sodium oxalate solution with 5 parts saline (0.15M sodium chloride), citrated saline by mixing one part sodium citrate with 99 parts saline.

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The following preparations were used as thromboplastins: (a) beef lung extract,[7] (b) ultracentrifuged beef lung extract,[8] (c) acetone dehydrated rabbit brain (Difco), (d) Russell viper venom (Stypven, Burroughs-Wellcome) (both [c] and [d] made up according to manufacturer's directions), (e) crude cephalin prepared from human brain,[9] 0.3 per cent suspension, (f) acetone dehydrated brain from both normal and hemophilic dogs and from a nonhemophilic human being, and (g) a synthetic cephalin, 1 per cent suspension.[11] Platelet suspensions contained about 1,700,000 platelets per cubic millimeter.[12] All dilutions were made with saline.

Accelerated clotting times, similar to prothrombin time determinations, were done at 37° C. by mixing 0.1 ml. oxalated or citrated plasma, 0.1 ml. thromboplastin preparation, and 0.1 ml. 0.02M calcium chloride.

Residual prothrombin in whole blood thirty and sixty minutes after venepuncture was determined by two procedures—the one-stage prothrombin consumption test[13] and the two-stage prothrombin utilization method.[14]

AHF was determined by two procedures: (a) a one-stage method described in the text of this paper, and (b) the prothrombin utilization method for the assay of AHF, described by Graham, Collins, Godwin, and Brinkhous.[15] Before use in either of these procedures, oxalated plasmas, both control and test, were treated with 100 mg. of barium sulfate per milliliter at 4° C.; the barium sulfate was kept in suspension for thirty minutes by gentle agitation, and then removed by centrifugation at about 3,000 *g* for thirty minutes.

Serum accelerator was prepared by the barium sulfate adsorption and elution method,[16] using as starting material native dog plasma which had been incubated for twenty minutes at 37° C. with 0.05 volume of beef lung extract. Concentration is expressed in units per milliliter.[16]

## RESULTS

*Comparison of Accelerated Clotting Times of Normal and Hemophilic Plasmas with Complete and Partial Thromboplastins.* The results of clotting time tests with the several thromboplastins are shown in Table I. Tests were done simultaneously with both normal (N) and hemophilic (H) plasmas, using for each thromboplastin a series of tenfold dilutions. The data given in the table are representative of a large group of determinations. It will be noted that similar results were obtained with human and canine plasmas. With the usual high concentrations of crude tissue extracts (Experiments 1 to 5, 9, and 10), normal and hemophilic plasmas have the same clotting times. These tissue extracts, diluted $10^{-2}$ to $10^{-3}$ or more, consistently clot the normal plasma more rapidly than the hemophilic plasma.

Ultracentrifugation of the tissue extracts (about 75,000 *g* for thirty minutes) caused sedimentation of potent thromboplastic material, while the supernatant fluid had little or no activity. The resuspended sediments were highly active (Experiments 6, 11, 12, in Table I), but even in high concentrations which gave clotting times of nine to twenty seconds, they had lost their ability to compensate completely for the hemophilic defect. Russell viper venom behaved similarly to the resuspended ultracentrifuged sediments (Experiments 8 and 14). Crude cephalin (Experiments 7 and 15) and platelets (Experiment 13) were comparatively poor in thromboplastic activity, but both were considerably more active in the clotting of normal plasmas. The weakest thromboplastin tested was a synthetic cephalin, L-$\alpha$-(dimyristoyl)-cephalin. It too showed a clear-cut differential in its acceleration of clotting of normal and hemophilic plasmas (Experiment 16). Only crude concentrated tissue extracts

were complete thromboplastins in the sense that they clotted normal and hemophilic plasma equally rapidly. All of the other thromboplastin solutions used were able to compensate only partially for the defect in hemophilia. We are calling the one-stage accelerated clotting time test, using such partial thromboplastins, the "partial thromboplastin time."

TABLE I. COMPARATIVE ACCELERATION OF CLOTTING OF NORMAL (N) AND HEMOPHILIC (H) PLASMAS BY VARIOUS THROMBOPLASTINS

| EXPERIMENT | THROMBOPLASTIN | PLASMA | THROMBOPLASTIN DILUTION | | | | | | SALINE |
|---|---|---|---|---|---|---|---|---|---|
| | | | $10^{0}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | |
| | | | *Group I. Human Plasmas* | | | | | | |
| | | | *Clotting Time (Sec.)* | | | | | | |
| 1 | Crude beef lung | N | 14.2 | 18.6 | 39.0 | 65.0 | 100 | 130 | 210 |
| | | H | 14.2 | 18.6 | 48.0 | 90.0 | 195 | 340 | 600 |
| 2 | Rabbit brain | N | 12.8 | 19.2 | 42.0 | 85.0 | 95 | 130 | 310 |
| | | H | 12.8 | 19.0 | 49.0 | 130.0 | 170 | 275 | 900 |
| 3 | Human brain | N | 13.2 | 18.6 | 40.0 | 80.0 | 90 | 120 | 250 |
| | | H | 13.2 | 18.8 | 45.0 | 120.0 | 160 | 280 | 780 |
| 4 | Normal dog brain | N | 17.2 | 24.6 | 42.0 | 60.0 | 85 | 110 | 195 |
| | | H | 17.2 | 24.2 | 42.0 | 80.0 | 140 | 235 | 840 |
| 5 | Hemophilic dog brain | N | 16.8 | 24.8 | 48.0 | 70.0 | 95 | 120 | 195 |
| | | H | 16.8 | 24.5 | 49.0 | 95.0 | 155 | 300 | 840 |
| 6 | Ultracentrifuged beef lung | N | 19.8 | 23.2 | 35.0 | 65.0 | 100 | 110 | 210 |
| | | H | 21.2 | 28.4 | 47.0 | 100.0 | 200 | 320 | 600 |
| 7 | Crude cephalin | N | 95.0 | 110.0 | 120.0 | 150.0 | 190 | 220 | 310 |
| | | H | 170.0 | 215.0 | 285.0 | 340.0 | 420 | 600 | 900 |
| 8 | Russell viper venom | N | 22.0 | 44.0 | 60.0 | 90.0 | 115 | 130 | 310 |
| | | H | 24.4 | 60.0 | 105.0 | 210.0 | 290 | 420 | 900 |
| | | | *Group II. Canine Plasmas* | | | | | | |
| | | | *Clotting Time (Sec.)* | | | | | | |
| 9 | Crude beef lung | N | 8.8 | 11.8 | 14.0 | 26.5 | 46 | 48 | 230 |
| | | H | 8.8 | 12.0 | 14.2 | 29.5 | 53 | 60 | 780 |
| 10 | Rabbit brain | N | 7.4 | 11.8 | 18.2 | 30.0 | 57 | 120 | 180 |
| | | H | 7.4 | 11.8 | 22.4 | 38.0 | 95 | 175 | 720 |
| 11 | Ultracentrifuged rabbit brain | N | 9.2 | 12.6 | 19.0 | 32.0 | 58 | 100 | 230 |
| | | H | 11.0 | 15.4 | 27.0 | 49.0 | 98 | 190 | 780 |
| 12 | Ultracentrifuged beef lung | N | 11.4 | 13.0 | 18.6 | 37.0 | 53 | 95 | 200 |
| | | H | 12.8 | 15.6 | 22.5 | 56.0 | 90 | 190 | 540 |
| 13 | Dog platelets | N | 45.0 | 48.0 | 57.0 | 60.0 | 65 | 85 | 190 |
| | | H | 85.0 | 90.0 | 95.0 | 100.0 | 125 | 165 | 620 |
| 14 | Russell viper venom | N | 14.8 | 28.0 | 43.0 | 56.0 | 80 | 100 | 184 |
| | | H | 17.2 | 35.0 | 65.0 | 115.0 | 140 | 210 | 660 |
| 15 | Crude cephalin | N | 37.0 | 44.0 | 50.0 | 58.0 | 80 | 100 | 210 |
| | | H | 85.0 | 95.0 | 115.0 | 200.0 | 270 | 400 | 730 |
| 16 | Synthetic cephalin | N | 52.0 | 65.0 | 75.0 | 90.0 | 115 | 130 | 155 |
| | | H | 145.0 | 170.0 | 190.0 | 235.0 | 295 | 470 | 840 |

*Presumptive Test for Hemophilia.*—The difference in clotting times of normal and hemophilic plasmas with partial thromboplastins gives a basis for a presumptive test for hemophilia. For the test, the prothrombin time and the partial thromboplastin time are determined for both a normal control plasma and the patient's plasma. The partial thromboplastin employed in this test may be any one of those listed in Table I. We have found it convenient to use Russell viper venom diluted $10^{-3}$ with saline. Acetone dehydrated human or rabbit brain is used as the complete thromboplastin.

The prothrombin time with the complete thromboplastin is dependent on the plasma for prothrombin, plasma accelerator(s), serum accelerator(s), and

fibrinogen. A normal prothrombin time indicates that none of these factors are seriously deficient. The partial thromboplastin time is influenced by these same clotting factors, and in addition by AHF. Thus a *normal* prothrombin time and a *prolonged* partial thromboplastin time furnishes presumptive evidence of hemophilia.

The usefulness of this test is illustrated in Table II. Data obtained on a normal control, a mild hemophilic, and a severe hemophilic patient are given. Three common tests used in the diagnosis of hemophilia—the whole blood clotting time, the one-stage prothrombin consumption test, and the two-stage prothrombin utilization test—showed marked delay in the rate of clotting in the classical hemophiliac. In the mild hemophiliac, on the other hand, all of these tests were normal. Yet the assay for AHF showed that the mild hemophiliac had only a trace of this clotting factor. The presumptive test for hemophilia was positive in both the mild and the severe cases of hemophilia, i.e., the prothrombin time was normal, the partial thromboplastin time was prolonged.

TABLE II. COMPARISON OF THE PRESUMPTIVE TEST FOR HEMOPHILIA WITH OTHER CLOTTING TESTS FOR HEMOPHILIA

| PATIENT | AGE | CLOTTING TIME (MIN.) | RESIDUAL PROTHROMBIN (PER CENT) | | | | AHF* (PER CENT) | PRESUMPTIVE TEST FOR HEMOPHILIA | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 30 MIN. | | 60 MIN. | | | PROTHROMBIN TIME (SEC.) | PARTIAL THROMBOPLASTIN TIME (SEC.) |
| | | | I STAGE | II STAGE | I STAGE | II STAGE | | | |
| Normal (RHW) | 31 | 10 | 62 | 20 | 18 | <5 | 100 | 12 | 120 |
| Mild hemophilic (HAD) | 69 | 10 | 58 | 12 | 15 | <5 | <5 | 12 | 300 |
| Classical hemophilic (TWT) | 26 | 60 | 152 | 100 | 146 | 92 | 0 | 12 | 350 |

*Prothrombin utilization method.[15]

*The Effect of Normal Plasmas on the Delayed Partial Thromboplastin Time of Hemophilic Plasmas.*—The relatively slow clotting of hemophilic plasmas with partial thromboplastins, as observed in Table I, can be corrected by the addition of small amounts of normal plasma. This is shown in two experiments, one with human plasmas, the other with canine plasmas, summarized in Fig. 1. Homologous normal and hemophilic plasmas were mixed in the ratios indicated (Fig. 1). Crude cephalin suspension was used as the partial thromboplastin. It will be observed that when the plasma mixture consisted of 15 to 20 per cent or more of normal plasma, the clotting time was normal. When the mixture consisted of smaller amounts of normal plasma, the impaired clotting of hemophilic plasma was partially corrected. We attribute the shortening of the hemophilic clotting to the antihemophilic activity (AHF) of the added normal plasma.

*A One-Stage Method for the Assay of Antihemophilic Activity.*—The assay is based on the observation that increasing amounts of AHF, within the limits noted in Fig. 1, caused progressive shortening of the partial thromboplastin time

of hemophilic plasma. By comparing the relative effectiveness of a known normal plasma and the patient's plasma, the AHF activity may be expressed as per cent of normal. In the assay, varying amounts of the material being tested for AHF are added to a constant amount of hemophilic plasma. The clotting times of the mixtures are then determined after addition of standard amounts of lipid thromboplastin and calcium chloride.

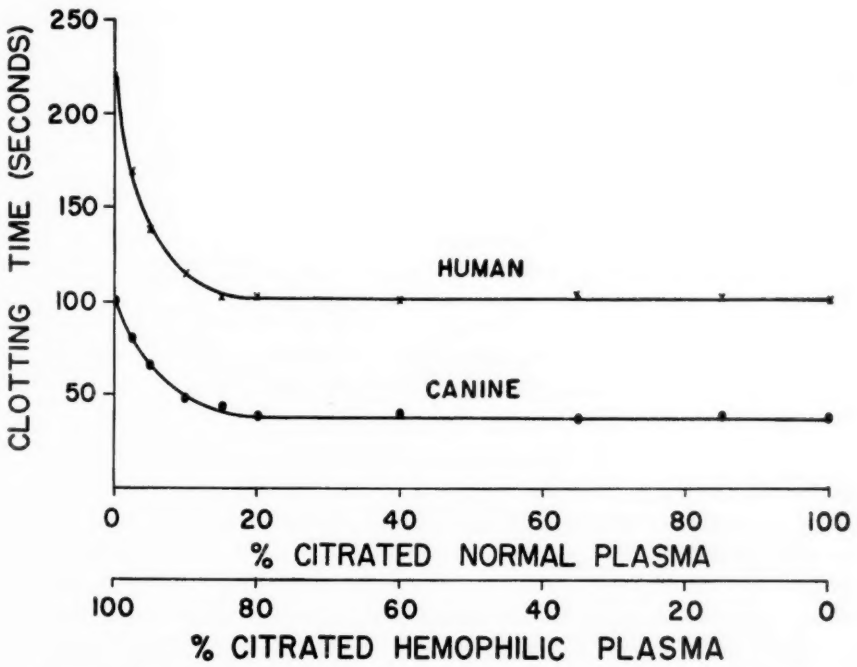


Fig. 1.—Partial thromboplastin times of mixtures of normal and hemophilic plasmas.

The details of the procedure follow. Citrated hemophilic plasma is used as a substrate; it may be stored up to thirty days at −20° C. or lower before use. Cephalin (0.015 per cent) is prepared from the 0.3 per cent stock suspension. The calcium-imidazole reagent consists of 7 parts 0.11M calcium chloride, 6 parts imidazole buffer,[17] pH 7.2, and 5 parts saline. The oxalated control and test plasmas are treated with barium sulfate and then diluted with oxalated saline to 1, 2.5, 5, and 10 per cent, respectively; higher concentrations for the test plasma are used when necessary. Prior dilution of the plasma with anticoagulant is used in calculating the amount of diluent to be added. During testing, the reagents are kept in a 28° C. water bath, except for the hemophilic plasma substrate, which is kept in an ice bath. For each clotting test the following are added, in the order indicated, to a 10 by 75 mm. dry glass tube: 0.1 ml. citrated hemophilic plasma, 0.1 ml. diluted control or test plasma, 0.1 ml. cephalin, and 0.1 ml. calcium-imidazole solution. Clotting times for each dilution of each plasma are determined in triplicate, and the mean determined. The mean clotting times are plotted against the per cent of the test or control plasma used, the latter being indicated on a log scale. By interpolation the dilutions of test plasma which would give the same clotting times as 1, 2.5, and 5 per cent control plasma are determined. The dilutions thus determined are used for the calculation of relative antihemophilic activity of the test plasma as follows:

$$\frac{\text{Per cent control plasma}}{\text{Per cent test plasma}} \times 100 = \text{per cent AHF activity in test plasma.}$$

Fig. 2 illustrates the procedure used for comparing the AHF in the test plasma with the control plasma. It will be noted that using the method of graphing indicated above, a linear relationship between clotting time and per cent plasma is obtained in this range. The test plasma used was from CBT, indicated below in Table V. The percentage values of test plasma which would give the same clotting times as 1, 2.5, and 5 per cent normal plasma are 1.4, 3.5, and 6.9 per cent, respectively. From these data a mean value of 71 per cent AHF in the test plasma is obtained.

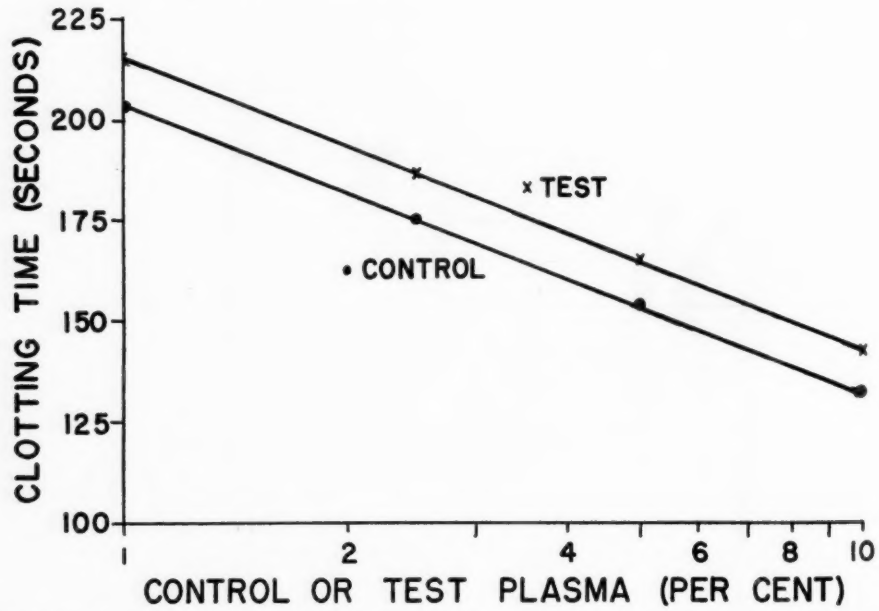


Fig. 2.—Graph illustrating method of plotting data for calculation of relative AHF activity in the one-stage assay.

*Influence of Thrombin and Serum Accelerator(s) on the Partial Thromboplastin Time and the One-Stage AHF Assay.*—Both thrombin and serum accelerator factor(s) are usually present in serum, and in addition, serum accelerator may evolve in plasma on standing.[18] To determine the influence of these clotting factors on the partial thromboplastin time, a series of experiments were performed in which each was added to the clotting mixtures. The data from one experiment are given in Table III. When the clotting times were shortened to the range of thirteen to seventeen seconds, the difference between normal and hemophilic plasma was nearly obliterated (Columns B and D). With lesser amounts of added thrombin or serum accelerator, the large spread in clotting times between the two plasmas was maintained (Columns C and E), even though clotting was considerably faster than that of the controls (Column A). In order to minimize the influence of such accelerators in uncontrolled amounts on the

TABLE III. INFLUENCE OF SERUM ACCELERATOR AND THROMBIN ON PARTIAL THROMBOPLASTIN TIME OF NORMAL AND HEMOPHILIC CITRATED HUMAN PLASMAS

| | CLOTTING TIME (SEC.) | | | | |
|---|---|---|---|---|---|
| | | THROMBIN UNITS/ML. | | SERUM ACCELERATOR UNITS/ML. | |
| | SALINE | 2.5 | 0.025 | 5.0 | 0.5 |
| PLASMA | A | B | C | D | E |
| Normal | 100 | 13.5 | 62 | 16 | 75 |
| Hemophilic | 265 | 15.5 | 140 | 17 | 180 |

Clotting mixtures: Cephalin (0.015 per cent), 0.1 ml. + calcium-imidazole solution, 0.1 ml. + saline, or dilute Topical Thrombin (Parke-Davis), or dilute serum accelerator preparation, 0.1 ml. + normal or hemophilic human citrated plasma, 0.1 ml. Units refer to concentration in final clotting mixtures.

AHF assay procedures, preliminary adsorption of the oxalated test and control plasmas with barium sulfate was done routinely. Barium sulfate adsorbs both the coagulant factors, thrombin,[19] and serum accelerator,[20] but does not adsorb AHF.[21]

In the one-stage assay, it was found that addition of thrombin or serum accelerator in the smaller amounts shown in Table III did not alter the results obtained for AHF with the procedure. The shorter clotting times which occur with the assay modified in this manner allow determinations for AHF to be made more rapidly. This practical advantage led to adoption of the modified procedure for clinical testing.

*Comparative AHF Values Obtained by the Two Methods of Assay; Range of AHF in Normal Individuals.*—In several experiments AHF activity was determined simultaneously by the prothrombin utilization method of Graham and associates and by the new one-stage procedure. Results of one experiment on protein fractions of normal canine plasma are shown in Table IV. Fractionation was carried out by simple precipitation of barium sulfate-treated oxalated dog plasma with neutral saturated ammonium sulfate at 4° C. By successive additions of the ammonium sulfate, precipitates were obtained which correspond to the following ranges of percentage saturation of salt: 0 to 25, 25 to 33, and 33 to 50 per cent. Phosphate buffer (pH 7.2) was used as solvent. These fractions and the final supernatant fluid were dialyzed, using Visking cellulose casing, at 4° C. with continuous agitation for two hours against citrated saline solution. In the calculation of AHF activity, dilution resulting from the frac-

TABLE IV. COMPARISON OF AHF IN FRACTIONS OF NORMAL CANINE PLASMA BY THE TWO-ASSAY PROCEDURES

| PLASMA FRACTION ($[NH_4]_2SO_4$ SATURATION RANGE IN PER CENT) | AHF ACTIVITY | |
|---|---|---|
| | NEW ONE-STAGE METHOD (PER CENT) | PROTHROMBIN UTILIZATION METHOD (PER CENT) |
| 0-25 | 91 | 70 |
| 25-33 | 18 | 14 |
| 33-50 | 11 | 8 |
| Above 50 | 0 | 0 |
| Total | 120 | 92 |
| Expected | 100 | 100 |

tionation procedure was considered. It is seen in the table that most of the AHF activity is precipitated at one-fourth saturation with ammonium sulfate. The values obtained by the two methods correspond fairly well.

In another experiment, freshly collected citrated plasmas of twelve normal male adults were tested for AHF by both methods. Results are shown in Table V. JBC was arbitrarily assigned a value of 100 per cent AHF. It will be noted that the values obtained on a given subject by either method correspond very well. However, there is a wide range of AHF in different individuals, from 64 to 138 per cent by the one-stage procedure, and from 67 to 136 per cent by the prothrombin utilization method. A larger group of normal adults of either sex was then tested with the one-stage AHF method alone. There were twenty-eight subjects. Again, JBC was assigned a value of 100 per cent. The range of AHF was 68 to 165 per cent, with a mean of 99, and a standard deviation of 28. These data indicate the need of careful selection of control plasma, if results are to be expressed as per cent of normal. We have consistently used either the plasma of a few individuals whose AHF level in relationship to the mean of a larger number of normals was known, or a pooled plasma from several healthy adults.

Further studies were made on the comparative AHF values in two normal subjects (GDP and RDL, Table V) over a period of twenty weeks. Ten separate determinations by the one-stage AHF assay were made during this period. The value of 72 per cent was assigned for the plasma AHF of GDP (Table V). The comparative AHF values for the plasma of RDL were 112, 107, 134, 111, 128, 122, 133, 119, 108, and 121, with a mean of 119 per cent.

TABLE V. COMPARISON OF AHF IN NORMAL SUBJECTS BY THE TWO ASSAY PROCEDURES

| SUBJECT | AHF ACTIVITY (PER CENT) | |
|---|---|---|
| | ONE-STAGE METHOD | PROTHROMBIN UTILIZATION METHOD |
| JPS | 64 | 70 |
| CBT | 71 | 65 |
| GDP | 72 | 67 |
| RHW | 80 | 79 |
| JPS | 81 | 89 |
| JBC | 100 | 100 |
| CA | 109 | 103 |
| WP | 124 | 112 |
| RDL | 120 | 114 |
| JBB | 132 | 124 |
| JBG | 138 | 123 |
| CP | 132 | 136 |

## DISCUSSION

The relative ineffectiveness of certain thromboplastins in the clotting of hemophilic blood provides the basis for both the presumptive test for hemophilia and the one-stage assay for AHF activity. It is not possible with the data now available to do more than postulate possible underlying reactions responsible for the differences in clotting of normal and hemophilic bloods with various thromboplastins. Biggs and Macfarlane[22] have shown that hemophilic plasma is less responsive to thromboplastin than is normal plasma, and is not simply deficient

in thromboplastin. Several years ago one of us[12] showed that both platelets and AHF are needed for thromboplastic activity to manifest itself in human blood. Similar results have been obtained in canine blood.[6] More recently comparable data were obtained by Shinowara[23] in a partially purified clotting system. The data in Fig. 1 are in accord with those obtained previously. All of these findings suggest that the full activity of potent crude tissue extracts on hemophilic plasma is due to their contamination with plasma AHF. However, one is then confronted with the paradox that hemophilic tissue extracts correct the hemophilic defect in vitro as well as do extracts from normal tissues (Table I). Instead, crude tissue extracts, both normal and hemophilic, may contain serum or other accelerators which obviate the need for AHF for rapid clotting in one-stage systems. This possibility is suggested by the data in Table III, in which a mixture of cephalin and a serum accelerator preparation became a potent clotting agent in the absence of AHF. Ultracentrifugation of the tissue extracts may effect a partial separation of thromboplastin from other clotting accelerators, and dilution may reduce their concentration to relatively impotent levels.

Regardless of the exact chemical interactions involved, the value of the two new procedures has been indicated during their use in this laboratory for the past two years. The simplicity of the presumptive test is of particular value in a clinical laboratory, and permits a rapid screening of patients suspected of having a bleeding diathesis. Mild hemophiliacs have been discovered by this method when other common clotting methods used for the diagnosis of hemophilia were negative (Table II). The more complex thromboplastinogen activity time, recently proposed as a diagnostic test for hemophilia,[24] appears to be based on the same mechanism as the presumptive test. While the presumptive test appears to be more sensitive than many of the other diagnostic tests, it has certain limitations, similar to those pointed out by Merskey[25] for other laboratory tests used in hemophilia.

A negative presumptive test was observed in a family with mild hemophilia, recently described by Graham, McLendon, and Brinkhous.[26] In this family the affected males had a plasma AHF activity of about 20 per cent of normal, and all had normal partial thromboplastin times. This was to be expected, however, since, as shown in Fig. 1, the clotting time of hemophilic blood is normal when 15 per cent or more of AHF is present.

A positive presumptive test appears to be given by at least one other bleeding disease besides hemophilia—PTC deficiency. This recently described hemorrhagic disease[27] appears to be due to a deficiency of a barium sulfate absorbable clotting component. While the prothrombin time is within normal limits in this disease, as in hemophilia, the partial thromboplastin time appears to be prolonged.[28]

A special problem is presented in the interpretation of the presumptive test if the prothrombin time is longer than normal. In such cases the partial thromboplastin time is also prolonged, without regard to AHF levels. To determine if a mixed deficiency exists, for example, a hypoprothrobinemia and

a lowered plasma AHF level, actual assays for these clotting factors must be performed.

The one-stage AHF assay has the advantage of simplicity, and appears to be as reliable as the more difficult prothrombin utilization method of Graham and associates. A possible drawback of both methods is that blood from a classical hemophiliac is a necessary substrate. This should not be a serious handicap in the one-stage assay, however, since the hemophilic plasma may be stored for considerable periods before use. Further experience with both of the new methods is needed to assess fully their value.


## SUMMARY

A comparison was made of the clot-accelerating activity of several thromboplastins on normal and hemophilic plasmas. The data indicate that thromboplastins may be classified according to their relative activity in the clotting of hemophilic plasma. *Complete* thromboplastins clot hemophilic plasmas as rapidly as they do normal plasmas, while *partial* thromboplastins clot hemophilic plasmas less rapidly than normal. One-stage clotting tests with the latter thromboplastins are called partial thromboplastin times, in contrast to prothrombin times, in which complete thromboplastins are used.

A presumptive test for the diagnosis of hemophilia is proposed. The test consists simply of the determination of a prothrombin time and a partial thromboplastin time. In hemophilia, the first time is normal, the latter prolonged. Some limitations of the procedure are discussed.

A simple method for the quantitative estimation of AHF by a one-stage clotting procedure is described. Hemophilic plasma is used as substrate. The method is based on the correction of the prolonged partial thromboplastin time of hemophilic plasma, by addition of small amounts of AHF. Application of this procedure to "salted out" plasma protein fractions revealed that nearly all of the AHF is precipitated by one-fourth saturation with ammonium sulfate. A study of the AHF activity of the plasmas of several normal subjects was made by the one-stage procedure. The AHF level of a given subject appears to be relatively constant. However, in different subjects, a range of activity was observed, with the maximum value being roughly twice the minimum.


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# LABORATORY METHODS

## METHOD FOR THE DETERMINATION OF ACETONE IN TWO-TENTHS MILLILITER OF BLOOD

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NUMEROUS methods can be found in the literature for the determination of acetone in blood.[1-4] Generally, the use of large quantities of blood is involved, or the preparation of a filtrate which leads to loss of acetone, or the method is time consuming.

The method of Rappaport and Baner[4] has the advantage of requiring only 0.2 ml. of whole blood without preparation of a filtrate. Two-tenths milliliter of whole blood is added to dilute phosphoric acid and the acetone in the blood distilled into iodine solution. The residual iodine is titrated with thiosulfate. In our hands several difficulties were experienced with this method. There was considerable variation in the blank even though the apparatus was thoroughly cleaned and some samples of normal blood gave titers indicating appreciable acetone. Also, with a quantity of acetone calculated to combine with all the iodine, a residual titer was obtained. For these reasons, the possibility of quantitatively distilling acetone from whole blood into a more specific trapping agent was examined. The method of Lester and Greenberg[3] using 2,4-dinitrophenylhydrazine as the trapping agent was combined with the distillation procedure of Rappaport and Baner.[4] In order to test the combination of these two methods the procedure detailed below, which also represents the final form of the method, was applied with the modifications necessary to test certain variables.

### METHODS

*Materials Required.*—

0.1 per cent 2,4-dinitrophenylhydrazine in 2N hydrochloric acid (hereafter designated as reagent).

Dilute phosphoric acid—1 ml. concentrated $H_3PO_4$ in 500 ml. distilled water.

0.3 per cent sodium bisulfite in distilled water.

0.5N sodium hydroxide.

Redistilled carbon tetrachloride.

Dow-Corning antifoaming agent A.

*Apparatus.*—

50 ml. glass-stoppered graduates.

50 ml. ₹ 19/38 Erlenmeyer flasks.

Glass rods—13 cm.

Colorimeter. The Evelyn colorimeter is used in this laboratory.

Special distilling apparatus (as modified from Rappaport and Baner[4]), Fig. 1.

From the Department of Physiology, Jefferson Medical College.
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*Procedure.—*

*Distillation:*

1. Pipette 2 ml. of reagent into a 50 ml. glass-stoppered graduate (Fig. 1, *F*). This is clamped to the apparatus so that the drawn-out tip of the condenser is under the surface of the liquid.
2. Pipette 2 ml. 0.3 per cent $NaHSO_3$ into the 50 ml. distilling flask. (Fig. 1, *A*.)
3. Pipette 0.2 ml. whole blood into distilling flask (*A*) and stopper.
4. Rinse the joint of the distilling head (Fig. 1, *B*) with distilled water.

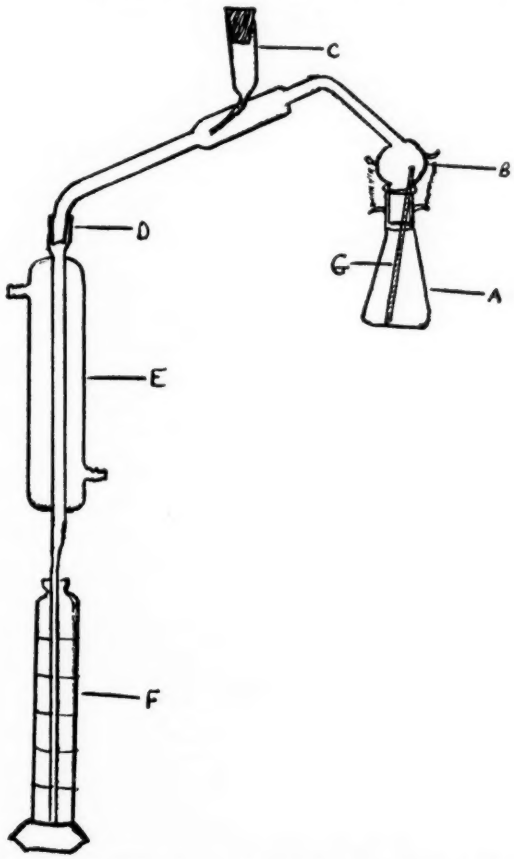


Fig. 1.—*A*, Distilling flask; *B*, distilling head; *C*, cleaning funnel; *D*, ₹ 19/38 joint; *E*, 200 mm. condenser; *F*, receiving flask; *G*, glass rod.

5. Place a glass rod (Fig. 1, *G*) with a dab of antifoaming agent on the end into the distilling flask (*A*).
6. Quickly add 5 ml. dilute $H_3PO_4$. Attach to distilling head and clamp.
7. Using a microburner, boil gently for five minutes, being careful to avoid foaming or boiling to dryness.
8. Lower the receiving flask (Fig. 1, *F*) and continue distillation for one minute longer. Remove flame.
9. Rinse several milliliters distilled water through cleaning funnel (Fig. 1, *C*) into receiving flask (*F*).

10. Remove receiving flask (*F*) and make up to 15 ml. with distilled water.
Wash apparatus between distillations by running through several hundred milliliters of distilled water with the aid of suction.

*Color Development:*

1. Add 10 ml. $CCl_4$. Stopper tightly and shake in mechanical shaker for fifteen minutes.
2. Draw off supernatant with gentle suction.
3. Add about 25 ml. distilled water and shake. Draw off water. Repeat wash.
4. Add 5 ml. 0.5N NaOH. Shake three minutes in shaker. Draw off NaOH.
5. Pour $CCl_4$ solution into colorimeter tube and read per cent T at 420 mu.

Prepare blank by distilling 2 ml. $NaHSO_3$ and 5 ml. $H_3PO_4$ into 2 ml. reagent and treat as if sample.

A standard is run the same as a sample, substituting a known quantity of redistilled acetone for the blood. 0.02 mg. acetone was found suitable for the standard.

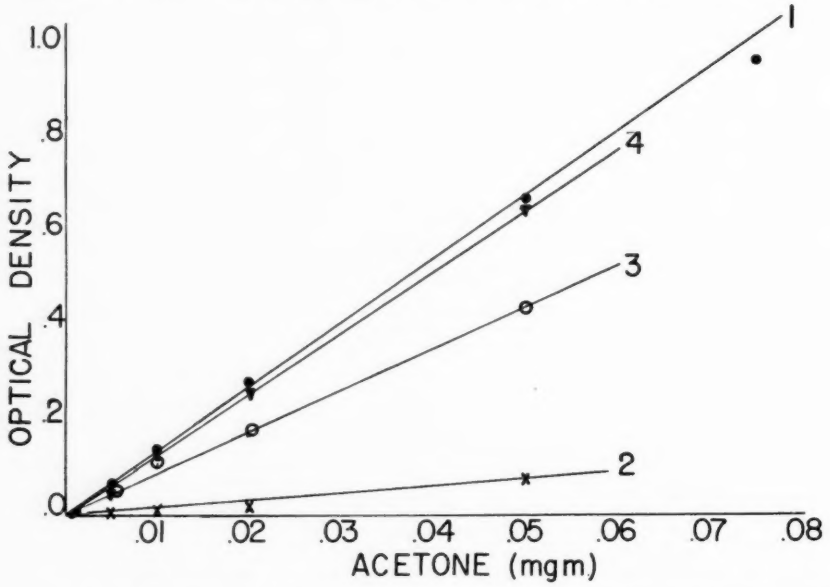


Fig. 2.—Extraction of acetone 2,4-dinitrophenylhydrazone under various conditions: *1*, dissolved in carbon tetrachloride; *2*, dissolved in 0.8 per cent hydrochloric acid; *3*, dissolved in 0.8 per cent hydrochloric acid, with 2 ml. reagent added to aqueous solution; *4*, dissolved in 0.8 per cent hydrochloric acid, with 4 ml. reagent added to aqueous solution.

## RESULTS AND DISCUSSION

It is known that the extraction of acetone 2,4-dinitrophenylhydrazone by carbon tetrachloride is subject to a number of variables.[3] In order to test the quantitativeness of the recovery of acetone 2,4-dinitrophenylhydrazone the pure compound was prepared. The melting point of this preparation was 125° C. (m.p. of pure compound—126° C.).

The above compound was made up in carbon tetrachloride and read at various concentrations at 420 mu and shown to follow Beer's Law (Fig. 2, Curve 1). If the extraction by carbon tetrachloride of the acetone 2,4-dinitrophenylhydrazone formed during reaction is complete, values of the order of these in Curve 1 should be obtained. However, a number of factors are found to influence the extraction and recovery of the acetone 2,4-dinitrophenylhydrazone formed.

Using the pure preparation again, but dissolving in distilled water with the aid of concentrated hydrochloric acid, then extracting with carbon tetrachloride (duplicating the test procedure) only 5 to 6 per cent can be recovered (Fig. 2, Curve 2). When 2 ml. of 2,4-dinitrophenylhydrazine reagent is added to the aqueous phase, however, approximately 70 per cent of the acetone 2,4-dinitrophenylhydrazone is recovered in the carbon tetrachloride (Fig. 2, Curve 3). The recovery can be increased to over 90 per cent using 4 ml. of reagent and shaking for fifteen minutes instead of ten minutes (Fig. 2, Curve 4), and can be made quantitative with 10 ml. of reagent.

It is obvious that an excess of reagent is necessary to carry the acetone 2,4-dinitrophenylhydrazone into the carbon tetrachloride. The use of large amounts of reagent, however, results in a very high blank (Table I), so that with samples of blood at or near normal levels such blanks introduce large errors.

TABLE I. VARIATION OF REAGENT BLANK

| ML. REAGENT | O.D. | ML. REAGENT | O.D. |
|---|---|---|---|
| 0 | 0.000 | 6 | 0.104 |
| 2 | 0.046 | 8 | 0.125 |
| 3 | 0.068 | 10 | 0.150 |
| 4 | 0.075 | | |

Test conditions: no acetone, 15 ml. volume of aqueous phase, 10 ml. $CCl_4$ ten minutes' shaking.

The recovery of acetone 2,4-dinitrophenylhydrazone is further influenced by the volume of the aqueous phase used (Table II) and the time of shaking. Increasing the time of shaking from ten minutes to fifteen minutes, keeping all other conditions constant, will enhance the color from 0.02 to 0.04 O.D. unit.

TABLE II. RECOVERY OF ACETONE 2,4-DINITROPHENYLHYDRAZONE FROM VARYING VOLUMES OF AQUEOUS SOLUTION

| VOLUME OF AQUEOUS SOLUTION | O.D. |
|---|---|
| 5 ml. | 0.236 |
| 10 ml. | 0.201 |
| 15 ml. | 0.168 |
| 20 ml. | 0.141 |
| 25 ml. | 0.125 |

Test conditions: 0.02 mg. acetone, 2 ml. reagent, 10 minutes' shaking.

The conditions for the test procedure can be chosen so that an absolute recovery approaching 100 per cent is achieved. For the purpose of the procedure, however, this is not essential. It is necessary only that the relative recovery be complete, i.e., a known quantity of acetone treated in the same manner will repeatedly give the same result. In this laboratory, we preferred to choose the conditions giving the least blank with the best extraction. Two milliliters of reagent is used in a volume of 15 ml. with fifteen minutes' shaking. Under these conditions, the recovery of acetone is complete whether added directly, distilled from aqueous solution, or from blood to which acetone has been added (Table III). The method will determine the acetone in blood ranging from 0 to 50 mg. per cent.

TABLE III. RECOVERY OF ACETONE

| MG. ACETONE ADDED | TO REAGENTS—NOT DISTILLED O. D. | TO REAGENTS—DISTILLED O. D. | TO BLOOD—* DISTILLED O. D. |
|---|---|---|---|
| 0.006 | 0.049 | 0.059 | 0.050 |
| 0.01 | 0.081 | 0.086 | 0.082 |
| 0.02 | 0.164 | 0.169 | 0.166 |
| 0.04 | 0.317 | 0.311 | 0.303 |

*O.D. given has been corrected for the blood alone which gave O.D. = 0.016.

## CONCLUSION

A simple, rapid method for the determination of acetone in 0.2 ml. whole blood is described.

The extraction of acetone 2,4-dinitrophenylhydrazone by carbon tetrachloride is subject to a number of variables, but, having selected suitable conditions, consistent results are obtained.

Acetone alone or acetone added to blood is recovered completely on distillation.

*Acknowledgments.*—I wish to express my sincere appreciation to Dr. I. J. Pincus for his help and encouragement in the development of this method, and to Dr. R. J. Rutman for the many valuable suggestions he made.

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# PROCEDURE FOR PRODUCTION OF ANTI-SHEEP HEMOLYSIN

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MANY procedures have been recommended for the production of anti-sheep hemolysin. We have attempted to produce hemolysin by numerous prescribed intravenous and intraperitoneal methods and have never been able to produce serum of high titer. Titers obtained ranged from 1-1000 to 1-8000 by Kolmer's methods of titration. Frequently not even a 1-1000 titer could be obtained, in spite of repeated attempts.

In 1932 when this Department undertook to standardize reagents for use in local public health department laboratories in this State, the need for production of a higher titered hemolysin than had heretofore been produced seemed essential. Tuft, Yagle, and Rogers,[1] in their experiments with typhoid vaccines, demonstrated a more persistent appearance of antibody response by the intracutaneous injection than by other methods.

Experiments were conducted in the production of anti-sheep hemolysin by employing the intracutaneous method. It developed that by injecting whole sheep blood intracutaneously into rabbits, followed by intravenous inoculation, surprisingly high titers resulted in over 75 per cent of the animals inoculated. It is not unusual to obtain a titer of 1-48,000 or higher. A titer of 1-20,000 proved to be average.

## METHOD

The following method of anti-sheep hemolysin production was initiated in April, 1933, in the California State Division of Laboratories and has been the standard procedure to date:

Six or eight medium-sized young rabbits weighing about 2 kilograms are given a series of five intracutaneous injections of whole sheep's blood every other day.

Two intravenous inoculations of 20 per cent washed sheep's cells which are thoroughly washed in 0.85 per cent physiologic saline to which 0.01 per cent magnesium sulfate[2] has been added are given, allowing three days to intervene between inoculations.

A trial bleeding is taken from the rabbit's ear on the third day after the second intravenous injection. If the titer is not sufficiently high, repeated intravenous injections are given.

Table I. Schedule of Injections

| DAY | DOSAGE IN ML. | ROUTE OF INOCULATION | MATERIAL INJECTED |
|---|---|---|---|
| 1 | 0.5 | Intracutaneous | Whole blood |
| 3 | 1.0 | Intracutaneous | Whole blood |
| 5 | 1.5 | Intracutaneous | Whole blood |
| 7 | 2.0 | Intracutaneous | Whole blood |
| 9 | 2.5 | Intracutaneous | Whole blood |
| 12 | 1.0 | Intravenous | 20% washed cells |
| 15 | 1.0 | Intravenous | 20% washed cells |
| 18 | Trial bleeding | | |

From the California State Department of Public Health, Berkeley, Calif., Division of Laboratories.

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If the titer is satisfactory, exsanguinate rabbit. Preserve the serum by adding equal parts sterile glycerin.

*Titration of Hemolysin.*—

1. 0.1 ml. of serum is mixed with 9.9 ml. of saline solution giving 1:100 dilution (or 0.2 ml. glycerinated serum plus 9.8 ml. saline).
2. Kolmer's[3] method of titration of hemolysin is employed.
3. For distribution purposes lots of hemolysin are mixed in the proper proportion to insure a titer of at least 1:10,000 when mixed with equal parts of C.P. glycerin.

## DISCUSSION

It has been our experience that sheep blood obtained from animals at a slaughterhouse produces as high a titer as sterile sheep blood. If the blood is badly contaminated the rabbit may develop ulcers at the site of inoculation. This condition, however, does not necessarily result in death of the animal nor in a hemolysin of low titer. Over a period of eighteen years 224 rabbits have been inoculated by the above procedure, resulting in an average titer of 1:20,000 by Kolmer's method of hemolysin titration.* Other laboratories have adopted this method as a routine procedure for years.† We have also tried this method of hemolysin production on one donkey and two horses. In one instance a 1-1000 titer was obtained in one of the horses. This observation is mentioned only as a possibility that a high titer might be produced in horses for large volume production of anti-sheep hemolysin.

## SUMMARY

This method, which has given consistently satisfactory results, is recommended for the production of a high-titered anti-sheep hemolysin.

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*Martha K. Hohl, Senior Bacteriologist in the Division of Laboratories, California State Department of Public Health, continues to employ this method, obtaining satisfactory results consistently.

†Meridian R. Ball, Assistant Professor of Bacteriology, Department of Bacteriology, University of California at Los Angeles.

# A SIMPLIFIED METHOD FOR THE DETERMINATION OF AMYLASE ACTIVITY IN SERUM AND URINE USING GLYCOGEN AND THE ANTHRONE REAGENT

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THE Somogyi method[1] for the determination of serum amylase has been widely used for many years. Recently Kibrick and associates[2] have reported a method which utilizes the anthrone reagent but retains starch as a substrate. Starch suspensions are unstable and require daily preparation. Complete removal of undigested starch is difficult. The use of glycogen as a substrate was investigated since it can be removed quantitatively by precipitation with methanol.

## MATERIALS AND METHODS

*Reagents.—*

Phosphate-sodium chloride buffer.[2]

Buffered glycogen substrate. Two grams of glycogen (Pfanstiehl C.P., specific rotation approx. +200 degrees) are dissolved in a solution containing 11 ml. of buffer and 89 ml. of water. The pH of this solution is 7.15 (Buffered glycogen substrate can be kept for at least a month in the refrigerator without precipitation, without change in pH, and with negligible hydrolysis.)

Absolute methyl alcohol.

Fuller's earth.

Standard amylase solutions. These solutions were prepared by diluting human pancreatic juice assayed by the method of Somogyi.

Anthrone reagent. 400 mg. of Microne (brand of anthrone, National Biochemical Co., Chicago) are dissolved in 200 ml. of 95 per cent sulfuric acid.

Standard glucose solutions. A stock standard glucose solution is prepared by dissolving 1.000 Gm. of glucose in 100 ml. of saturated benzoic acid. The working standard glucose solution is prepared by diluting 1.00 ml. of the stock standard to 100 ml. with distilled water. Both solutions are kept in the refrigerator.

## PROCEDURE

Three milliliters of glycogen substrate are placed into each of two test tubes, labeled No. 1 and No. 2, respectively. The tubes are placed into a 37° C. water bath for several minutes. 0.20 ml. of serum or urine is added to tube No. 1, and the contents are mixed by agitation. Incubation is continued for exactly fifteen minutes. At the end of this time, 3.00 ml. of absolute methyl alcohol are added to both tubes, followed by 0.20 ml. of serum or urine to tube No. 2. The tubes are corked with rubber stoppers and shaken for thirty seconds. Two grams of Fuller's earth are added, and the tubes are shaken vigorously two minutes and centrifuged.

After centrifugation, 0.40 ml. of supernatant from each tube (or 0.20 ml. plus 0.20 ml. of water if high values are expected) is transferred into a Klett colorimeter tube correspond-

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ingly labeled. 1.60 ml. of water and 4.00 ml. of anthrone reagent are added. The contents are mixed by stirring with a glass rod. A tube containing 0.40 ml. of working standard glucose solution and a blank containing 0.40 ml. of water are set up in a similar manner. After ten minutes the color intensity of all tubes is measured in a Klett-Summerson colorimeter using the red filter and setting the colorimeter to zero with the blank.

In order to permit a direct utilization of the colorimetric readings, units of amylase activity are defined as follows: one hundred units of amylase activity are present in one hundred milliliters of fluid when the quantity of liberated anthrone-reacting substance present in 0.40 ml. of supernatant gives the same reading as 0.040 mg. of glucose. Units of amylase activity are thus calculated as follows:

$$\text{Glycogen-anthrone Units of Amylase Activity} = \frac{\text{Reading of tube No. 1} - \text{Reading of tube No. 2}}{\text{Reading of standard glucose solution}} \times 100.$$

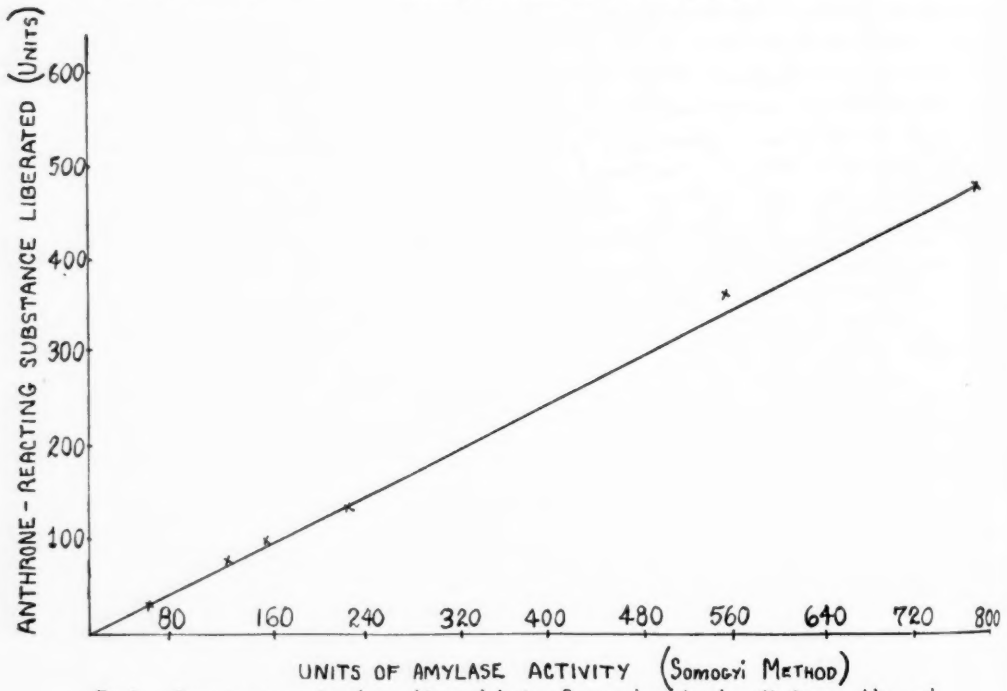


Fig. 1. Comparison of values obtained by the Somogyi method with those obtained by the glycogen-anthrone method using standard amylase solutions.

## RESULTS

Standard amylase solutions were assayed by the glycogen-anthrone method and by the Somogyi method. A linear relationship was found to exist between glycogen-anthrone units of amylase activity (units of anthrone-reacting substance liberated) and enzymatic activity as determined by the Somogyi method up to 800 Somogyi units (Fig. 1). If a value exceeding this level is obtained, the procedure should be repeated using 0.10 ml. of enzyme-containing solution and 0.10 ml. of saline.

A linear relationship exists between time of incubation and glycogen-anthrone units of amylase activity (Fig. 2). A fifteen-minute incubation period

was selected for routine determinations since sufficient enzymatic activity occurs in this time to allow for satisfactory colorimetric determination.

In Table I units of enzymatic activity obtained by the Somogyi method are compared with those obtained by the glycogen-anthrone method. Twenty specimens of serum and urine were assayed by the glycogen-anthrone method and the Somogyi method. A consistent correlation was observed and a mean conversion factor of 1.62 was obtained:

Somogyi units = 1.62 × glycogen-anthrone units.

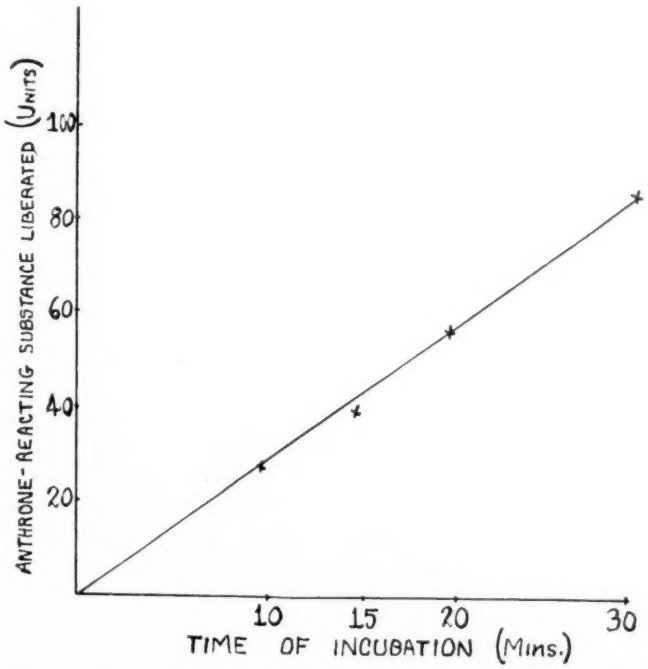


Fig 2. Relation between time of incubation and units of anthrone-reacting substance liberated.

## DISCUSSION

The glycogen-anthrone method permits an accurate assay of amylase activity in a relatively short time. Only 0.40 ml. of serum or urine is necessary in contrast to larger quantities demanded by other methods.

The combined glycogen-buffer solution offers several advantages. It is easily prepared and can be kept for at least a month in the refrigerator without precipitation, without change in pH, and with negligible hydrolysis. Starch suspensions require daily preparation.

A glycogen-anthrone unit of amylase activity as defined has been found to be 0.617 of a Somogyi unit. By use of the factor 1.62, glycogen-anthrone units can be converted to Somogyi units.

TABLE I. COMPARISON OF VALUES OBTAINED BY SOMOGYI METHOD WITH THOSE OBTAINED BY THE GLYCOGEN-ANTHRONE METHOD

| PATIENT NO. | SOMOGYI (UNITS OF AMYLASE ACTIVITY) | GLYCOGEN-ANTHRONE (UNITS OF AMYLASE ACTIVITY) | CONVERSION FACTOR (GLYCOGEN-ANTHRONE TO SOMOGYI UNITS) |
|---|---|---|---|
| 1 (Serum) | 128 | 78 | 1.64 |
| 2 (Serum) | 315 | 189 | 1.60 |
| 3 (Serum) | 396 | 240 | 1.65 |
| 4 (Serum) | 66 | 41.5 | 1.59 |
| 5 (Serum) | 50 | 30 | 1.67 |
| 6 (Serum) | 55 | 33 | 1.66 |
| 7 (Serum) | 59 | 34.5 | 1.71 |
| 8 (Serum) | 38 | 23 | 1.65 |
| 9 (Serum) | 104 | 66 | 1.58 |
| 10 (Serum) | 124 | 77.5 | 1.60 |
| 11 (Serum) | 200 | 125 | 1.60 |
| 12 (Serum) | 172 | 96 | 1.79 |
| 13 (Serum) | 122 | 84 | 1.45 |
| 14 (Serum) | 37 | 23 | 1.61 |
| 15 (Serum) | 53 | 30 | 1.77 |
| 16 (Urine) | 117 | 74 | 1.59 |
| 17 (Urine) | 159 | 106 | 1.50 |
| 18 (Urine) | 290 | 194 | 1.49 |
| 19 (Urine) | 35 | 22 | 1.60 |
| 20 (Urine) | 235 | 149 | 1.58 |
| | | | 1.62 ±0.07* |

*Standard deviation $= \sqrt{\frac{\Sigma\ d^2}{n-1}}$


## SUMMARY

A simplified method is described for the determination of amylase activity in serum and urine. After incubation with buffered glycogen solution for fifteen minutes, methyl alcohol is added to precipitate the remaining glycogen. The anthrone-reacting substance liberated is determined colorimetrically. Results obtained are correlated with values obtained by the Somogyi amylase method.


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# A METHOD FOR THE DETERMINATION OF THE EFFECT OF HYPOTHYROIDISM ON GASTRIC MOTILITY IN THE DOG

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## INTRODUCTION

IN THE course of observations on patients in the Ohio State University Hospital, an apparent relationship was seen between abdominal pain and gastric hypermotility in individuals with hypothyroidism.[1, 2] Because of the difficulty of obtaining controlled observations in human subjects, an attempt was made to make similar studies in dogs. These studies are not yet completed, but the method has proved useful in our laboratories. As very little work has been done on this subject in the past, the techniques used are discussed at this time that their simplicity may aid others who wish to make similar studies.

## MATERIALS AND METHODS

The procedure consists of gastric motility studies on the unanesthetized dog, with simultaneous respiratory tracings, a modified somnolent metabolic rate determination, and blood iodine and serum cholesterol determinations. A record of the pulse rate and weight is kept. The dogs are then subjected to a total thyroidectomy with preservation of the parathyroids, and the same determinations made at intervals postoperatively.

1. *Gastric Motility Studies.*—The most simple type of apparatus was considered to be the use of an intragastric balloon, with bromoform manometers and ink-writing floats recording on a dual-drum kymograph.

A Miller-Abbott tube was shortened to serve as a gastric tube. In order to permit maximum accuracy and to render tracings as quantitative as practical, there are several factors to consider in preparing these tubes. The diameter and length of the Miller-Abbott balloon should remain uniform and be recorded with each tracing. These measurements are made as follows: The *diameter* is most accurately measured before the balloon is attached to the tube. This avoids inaccuracy due to stretching when the balloon is inflated. The *length* of the balloon is the distance between the proximal and distal tie holding the balloon on the tube. These precautions are taken because it is felt that if balloons with the same diameters and lengths are filled with approximately the same volume of air, the resulting tracings can be compared more accurately. This takes into consideration the point made by MacQuigg,[3] who illustrated the difference that the size and shape of the balloon can make. Thus two balloons of different sizes can be filled with an equal volume of air, but the shorter one is more likely to record only one peristaltic wave at a time. The longer balloon, by recording two waves simultaneously, can produce a tracing showing a false summation-type of contraction.

The *volume* of air used in the balloon is recorded so that subsequent tests can be done under conditions similar to the original tracings. This is particularly important in serial studies on the same animal.

The fourth measurement to be recorded is the *distance* the tube is down. The position of the tube is checked by x-ray at the end of the first test on each animal. No contrast

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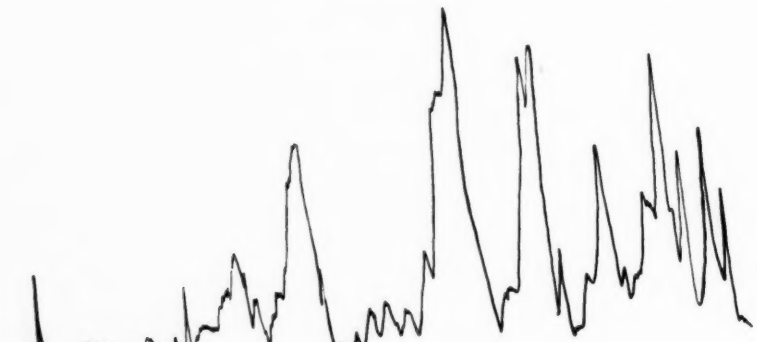
medium is needed for this, as the relationship of the metallic tip to the gastric air bubble is deemed adequate for these studies. The tube being held by tape and marked at a known point, such as the level of the incisor teeth, during the motility tracings and the x-ray, it can be replaced to approximately the same portion of the stomach on subsequent examinations without the need for more than one x-ray study on each dog.

The introduction of the tube into the stomach of the intact fasting unanesthetized dog was found to be relatively easy. The mouth of the animal is tied over a rectangular rubber block which is initially placed sufficiently far back to hold the mouth open widely. The deflated balloon is lubricated with a water-soluble lubricant. An assistant studies the dog



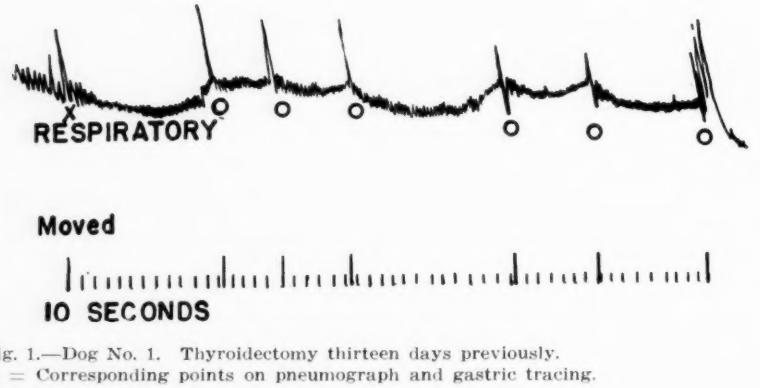




Fig. 1.—Dog No. 1. Thyroidectomy thirteen days previously.
X = Corresponding points on pneumograph and gastric tracing.
Dog, dozing quietly.
O = At height of gastric contraction, awoke whimpering then immediately dozed again.

while the balloon is thrust through a hole in the center of the block into the pharynx. It was found that the passage of the balloon into the pharynx was facilitated by applying gentle traction to the tongue. The tie is loosened, and the block moved forward in the mouth to allow the mouth to be partly closed to a position in which the animal is more comfortable and can swallow more easily. The block is then tied securely in place with gauze bandage. The balloon is introduced to the desired level as the dog swallows, and anchored in position by means of adhesive tape. It has been shown that in the dog, as in man, gastric motility is affected by the emotional state of the individual, and various environmental factors.[4] Therefore the animal is handled as gently as possible, and the tests are

conducted in a quiet, isolated room. Unlike the technique of MacQuigg and Kingma,[5] the dog sits or lies on a table with the assistant sitting beside him, no restraints being used other than the assistant's hand.

Although Carlson's studies incorporated simultaneous pneumographic tracings, a number of investigators since have neglected to do so. A tracing of pseudo-gastric hypermotility can be obtained by various extragastric movements. Usually the experienced observer can distinguish artifacts from true gastric contractions, but we have observed instances of artifacts on the gastric tracing resulting from body motion or hyperventilation mimicking types II and III gastric contractions. Therefore, in order to record the motions of the thoraco-abdominal wall simultaneously with the intragastric contractions, a balloon was attached to a rectangular piece of firm rubber webbing and bandaged firmly in place over the chest. The single-lumen tube from this balloon is attached to an ink-writing bromoform manometer in the same manner as that from the gastric balloon. Time is recorded by means of a signal magnet marking ten second intervals. All tracings are of at least one hour duration so that the initial part of the test which might be influenced by excitement incident to the passage of the tube, and by the presence of the small amount of lubricant, can be discounted, and so that the pattern of contractions can be observed. Fig. 1 is a photograph of a portion of a tracing obtained from a very lethargic thyroidectomized dog whose metabolic rate had dropped from a pre-operative level of +16 to −3 per cent. The value of the respiratory tracing is seen as it illustrates the relationship of the motion of the otherwise quiet animal to the high gastric contractions.

2. *Metabolic Rate Determination.*—The metabolic rate of the dog is determined under intravenous Nembutal anesthesia in the fasting state. Nembutal has been shown by Cavett[6] to have no significant effect on the basal metabolic rate of dogs. Its use obviates the necessity of training the animal, and removes the unmeasurable influence of environmental stimuli. The dosage of Nembutal is not predetermined, the drug being injected fairly slowly in the concentration of 60 mg. per milliliters until the dog does not react to tactile stimuli, usually about 4 to 6 ml. being required. To minimize the amount of Nembutal required, and to reduce reflex respiratory effects, the larynx and trachea are then anesthetized with 1 per cent pontocaine in the form of a spray supplemented by its application by means of a cotton swab. The largest possible endotracheal tube is inserted. A size 38 Magill tube with an inflatable cuff has been found satisfactory in most cases to enable the maximum respiratory exchange without leakage. The tube is attached to a standard basal metabolism machine, both the Benedict-Roth and McKesson machines having been used in this laboratory and found satisfactory for this purpose. The only adaptation needed to convert the machine from use for human tests to use for dogs was found to be the necessity for the shortest possible rubber hose between the machine and the mouthpiece connection so as to minimize the dead space. The mouthpiece used for human B.M.R. determinations served as a completely adequate adapter, being attached to the machine in the usual way, with the proximal end of the endotracheal tube inserted into the lumen of the mouthpiece in the portion of the mouthpiece which is ordinarily inserted into the patient's mouth. The machine is then operated as for a human B.M.R. determination. If the animal has regular, normal respirations and the tracing shows the low uniform oxygen consumption characteristic of a satisfactory test, a three-minute record is entirely adequate.

In calculating the basal metabolic rate of dogs, there are two methods which may be applied. One is to base the determination on the *surface area,* as is ordinarily done in man. Probably the most widely used formula for this is the Cowgill-Drabkin formula,

$$\text{S.A.} = 2.268 W_{\text{Gm.}}^{0.367} L_{\text{cm.}}$$

S.A. being the surface area in square centimeters, W the weight in grams, and L the length of the dog from tip of nose to anus in centimeters.[7]

Comparing the results obtained in this way with those obtained on the basis of *weight,* the latter appeared, in our observations, to be much more accurate. This agreed with the

findings of Brody, who feels that in animals the weight basis is to be preferred.[8] We therefore determine the theoretical normal for the dog using Brody's formula:

$$Q = 70.5M_{kg}^{0.734}$$

Q being the number of calories produced per twenty-four hours, and M the weight of the animal in kilograms.[8] The percentage difference between this theoretical normal and the value obtained experimentally is the metabolic rate as recorded in our results.

3. *Laboratory Studies.*—The serum cholesterol and blood iodine determinations are made in the usual manner. We have observed that some commercial dog foods have an exceedingly high iodine content, giving soluble blood iodine fractions of about 30 mcg. per cent and completely invalidating protein-bound iodine determinations. Therefore, unless the composition of the commercial preparation is definitely known, these should be avoided.

## SUMMARY

A simple plan for the determination of the effect of hypothyroidism on gastric motility in the dog is presented. The methods described are readily applicable to other studies of gastric physiology and metabolism in the dog. The equipment used is that readily available in a general hospital with access to physiological laboratory facilities.

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